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The
BACTERIAL CELL

*In Its Relation to Problems of
Virulence, Immunity and Chemotherapy*

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En souvenir de nos rêves

IN APOLOGIA

L'HOMME DE RECHERCHE entraîné à la poursuite d'un problème particulier n'a pas à se préoccuper, autant que dure son effort, du problème général de la science. Ses investigations se concentrent sur un point limité; et pendant qu'il s'occupe à sa tâche dans un coin de l'édifice que la science contemporaine élève avec tant de rapidité, il n'est pas nécessaire qu'il embrasse le plan de cet édifice auquel collaborent tant d'autres études que les siennes. Cependant c'est à réaliser ce plan qu'il travaille d'une manière consciente ou inconsciente, comme maçon ou comme architecte.

Il n'y a donc rien de plus profitable pour un esprit philosophique et généralisateur que de chercher à pénétrer ce dessein qui se réalise par suite de l'évolution naturelle et fatale de la science. C'est ce que nous essayerons de faire ici même. De telles tentatives offrent le double avantage de satisfaire à un besoin de l'intelligence et de contribuer à l'avancement de la science.

CLAUDE BERNARD

PREFACE

J'aime mieux être homme à paradoxes qu'homme à préjugés.

JEAN JACQUES ROUSSEAU

THE microorganisms classified as bacteria probably contain representatives of several unrelated biological groups. In addition to physicochemical properties shared by all living forms, each bacterial type possesses a structural and biochemical individuality which could serve as a basis for an orderly statement of the problems of cellular organization, and for a rational system of classification based on phylogeny. Unfortunately, known facts concerning these distinctive characteristics of each bacterial type are too few and too unevenly scattered among the different microbial groups to lend themselves to convincing integration and generalizations. This situation is due to the fact that, in general, most microorganisms have been studied only from the point of view of their importance in practical problems, and usually without much regard to the other aspects of their biology and chemistry, or to the homologous characteristics of related strains.

For example, the bacteria utilized in the fermentation processes and those which are of importance in the economy of organic matter, or capable of performing interesting and unusual chemical reactions, are studied very largely from the point of view of biochemistry. It is possible to describe their behavior as catalysts of metabolic systems without so much as mentioning their structure as cellular organisms. This approach has given rise to a school of chemical bacteriology which utilizes the methods and points of view of intermediary metabolism and which, in reality, is a part of classical biochemistry.

The study of pathogenic bacteria has proceeded along entirely different lines. In order to analyze the host-parasite relationship,

the student of infection concerns himself primarily with those structures and products of bacteria—the cellular antigens and toxins—which affect the course of the infectious process and against which are directed the reactions of immunity. Many constituents of the bacterial cell have been recognized first by their biological activity. Analysis of the phenomena of infection, immunity, and chemotherapy has thus provided important information concerning bacterial cytology.

A considerable body of knowledge concerning the biological and chemical architecture of bacteria is slowly emerging from these indirect methods. It will be the purpose of the present book to integrate this information with the data obtained by the classical techniques of cytology, and to interpret some of the phenomena of the infectious process in terms of the bio-chemical architecture of the bacterial cell.

No attempt will be made to present a survey of all groups of bacteria, or an exhaustive study of any one particular group. Examples will be selected not on the basis of the importance of the organism concerned, but only with regard to the extent and interest of information available, relevant to the problem under discussion. It has been judged inadvisable in most cases to develop the subject along historical, chronological lines, and, consequently, many investigations have not been quoted, even when their results are obviously incorporated in the text. Whenever possible, reference has been made to recent reviews presenting extensive bibliographies of the subject.

The present volume is the outgrowth of a course of eight lectures delivered during February, 1944 under the auspices of the Lowell Institute in Boston. I wish to express to the trustees of this Institute my gratitude for giving me the opportunity to put into execution a project first outlined in 1938 in the course of conversations with Dr. R. H. S. Thompson, now of the Biochemical Laboratory at the University of Oxford, England. It would be impossible to mention and to thank all those who, directly or indirectly, have helped in the preparation of the manuscript, and who have given permission to reproduce the data and illustrations incor-

porated in the text. I owe much also to Miss Catherine M. Casassa, Miss Jean Porter, and Mrs. Elizabeth Fuller of the Department of Comparative Pathology and Tropical Medicine, Harvard Medical School and School of Public Health. Miss Casassa managed to establish and to maintain order in the shapeless mass of manuscript and references which I submitted to her. Miss Porter read the whole manuscript in an attempt to conceal the foreign flavor of my English style. In addition to sheltering me from many administrative problems by her efficient and good-natured management of the Department, Mrs. Fuller valiantly helped us during many demoralizing June days.

Those who have been at some time connected with the Hospital of the Rockefeller Institute will undoubtedly hear through the following pages the echoes of many a conversation and discussion held at the Monday Night Journal Club and especially in the laboratories of the Division of Respiratory Diseases. I shall be rewarded for my efforts if this book brings back to their memory—even though in a blurred and distorted manner—the smiling wisdom of one known to all for his great scientific achievements, and whom so many call with admiration, gratitude, and love “The Professor”—Dr. Oswald T. Avery.

Boston, Massachusetts
June, 1944

RENÉ J. DUBOS



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THE BACTERIAL CELL

THE BACTERIAL CELL

reduction of the latter the energy released by the oxidation of inorganic substances—ammonia, nitrites, sulfur, etc.—(van Niel, 1943). Could not this most primitive biochemical expression of life, the production of organic matter purely from inorganic substances, be considered as the beginning of LIFE on earth?

Cytological studies were quick to dispel any illusion as to the structural simplicity of bacteria. Differential staining reactions, study of sporulation and of spore germination, analysis of osmotic behavior, and even microdissection experiments revealed the existence in bacteria of various kinds of intracellular bodies, organs of locomotion, plasma membranes, cell walls and capsules, etc., which give to the morphology of each bacterial type a characteristic complexity (Chapter II). Enough knowledge had accumulated to justify in 1912 the publication of a monograph, *Die Zelle der Bakterien*, in which A. Meyer limited his discussion to the cytology of the bacterial cell. Evidence of the complexity of bacteria has continued to accumulate as new techniques have been introduced, such as an increase in the resolving power of microscopy, the use of staining reactions endowed with chemical specificity, or the analysis of cellular organization by indirect methods based on physiological behavior. Thus, new structures have been revealed and the existence of others foreshadowed. At the cytological level at least, the morphology of bacteria may not differ essentially from that of plant and animal life (Chapters III and IV).

During the same time, the biochemist has come to recognize in microbial life the same chemical reactions, the same metabolic channels and products, and the same biocatalysts which constitute the mechanism of organic life in the highest organisms (Barron, 1943; Werkman and Wood, 1942). Even the autotrophic bacteria, those "primitive" beings capable of synthesizing life from the atmosphere and the rock, are now shown to operate through the same elaborate mechanisms characteristic of the most evolved metabolic types. The autotrophic oxidation of sulfur by *Thiobacillus thiooxidans*, for instance, depends upon an intimate linking between oxidation and phosphate turnover; the oxidative phase is



FIG. 1.—Electron micrograph of partial ghost of *A. fischeri* in distilled water. It appears that the cell wall has peeled off of approximately one-half the cell, leaving the naked protoplast, with flagella attached at one end. The thin, crumpled structure is the cell wall, and for this possibly represents a portion of the wall that has come off. Note that the diameter of the flagella varies, according to recognizable differences in the number of longitudinal components in certain regions (From Johnson, Zworykin, and Warren, 1943, fig on p 171)



FIG. 2.—Electron micrograph of *B. cereus*.
(From Johnson, 1944.)

accompanied by phosphate fixation, and the reductive phase of carbon dioxide fixation by a release of phosphate (Vogler, *et al.*, 1942; Le Page, 1942). The same organism is fully equipped with the regular complement of water soluble vitamins found in other organisms: thiamin, riboflavin, nicotinic and pantothenic acids, pyridoxin and biotin (O'Kane, 1942). Not only do autotrophs utilize these multiple and complex biocatalysts, but they also have the ability to synthesize them from inorganic elements, a property which most plant and animal cells either never possessed, or have lost. The high degree of biochemical organization required for the performance and the integration of these complex syntheses need not be emphasized; neither is it surprising that electron microscopy should reveal in the cells in which they take place a number of structures, often ill defined in their nature and functions, but expressing a morphological complexity which parallels biochemical complexity (Knaysi, 1943). The growth requirements of autotrophic bacteria are extremely simple indeed, but how complex their vital machinery, their performance, and their products! If they are truly the first representatives of life on earth, they sprang, like Minerva, fully armed from the forehead of Jove.

In the following chapters, many other illustrations will be given of the structural and chemical complexity of bacteria. It will be shown also that their biological behavior reveals a pattern of organization very similar to that of plant or animal cells. Like the latter, they give rise to mutation-like phenomena at an approximate rate of 1 per 10^5 to 10^6 cellular divisions; their growth is governed by laws which recall those governing the growth of the multicellular organisms since they exhibit in turn an embryonic, a mature and a senescent form (Chapter V:2). In reality it appears, therefore, that it is only their small size and the absence of recognized sexual reproduction which has given the illusion that bacteria are "simple" cells. Failure to recognize a multiplicity of structures underlying the multiplicity of functions should be regarded as an indication of the deficiency of our experimental techniques, rather than as evidence of a simplicity of cellular organization.

2. BIOLOGICAL NATURE AND PHYLOGENY OF BACTERIA

Let all things be done decently, and according to order.

ST. PAUL, I CORINTHIANS, XIV, 40

Natura non facit saltus.

GOTTFRIED WILHELM VON LEIBNITZ

Recognition of the complexity of bacteria—both structural and biochemical—explains why the early attempts to consider them as starting points for evolutionary systems were soon abandoned. On the other hand, the diversity of their structure and behavior seems to render impossible any definition which would include all the microbes commonly recognized as bacteria, and exclude at the same time those which clearly belong to other well defined divisions. One finds among bacteria organisms which show strong resemblances to certain of the blue-green algae, to the fungi, to the myxomycetes, or to the protozoa, and which can only be distinguished from these microorganisms by their much smaller size.

Some investigators have looked upon bacteria as a primitive homogeneous group from which higher types have arisen. It appears more likely, however, that these microorganisms constitute a heterogeneous group of unrelated forms. Even among the Eubacteriales—the so-called true bacteria—one finds strange bedfellows, such as small Gram-negative autotrophic organisms, the Gram-positive proteolytic spore formers, the acid-fast bacilli, which differ so profoundly from each other in metabolism, structure, and even mode of division as to have little in common except microscopic dimensions. One may indeed wonder whether the apparent unity of the group is not due to a narrow range of cellular size which determines, by a sort of convergent evolution, a number of physical and chemical characteristics. These in their turn have imposed certain experimental disciplines and techniques which define the methods employed by bacteriologists rather than the biological material which they study.

In the classification of higher plants and animals, systematists have relied almost exclusively on morphology, special attention being paid to the reproductive structures. In bacterial systematics,

on the contrary, extensive use of physiological criteria has been made—a course imposed in part by the scarcity of morphological data. It is unlikely, however, that the distribution of metabolic characters can reveal the trends of physiological evolution, and, for the present time, any attempt to develop a phylogenetic system will have to be based to a large extent on what morphological characters are available. Among these can be mentioned the nature of the cell wall, the presence and location of chromatin material, the functional structures (e.g., of locomotion), the method of cell division and the shape of the cell, the type of organization of cells into larger structures, and the nature and structure of reproductive or resting cells (Stanier and van Niel, 1941). It is possible that, eventually, serological reactions can be used to establish relationships between the immunochemical specificities of homologous cellular constituents of different microbial species, and thus add an independent line of evidence to any phylogenetic system (Chapter IV: 2); unfortunately, only scattered data are available concerning these comparative serological reactions.

Granted the paucity of information, it may be useful to present the following tentative outline which describes the relationships within the kingdom *Monera*, i.e., microorganisms without true nuclei, plastids, and sexual reproduction (Stanier and van Niel, 1941).

- A. Photosynthetic organisms evolving oxygen and possessing the typical green plant chlorophylls, phycocyanin and sometimes phycoerythrin, and colorless, non-photosynthetic counterparts, clearly recognizable as such.
 - Division I *Myxophyta*

- B. Organisms not so characterized.

- I. Unicellular or mycelial organisms with rigid cell walls.
 - Division II *Schizomycetae*

Motility, when present, by means of flagella. Endospores, cysts, or conidia may be formed. Class 1 *Eubacteriae*

- a. Organisms photosynthetic, but not producing oxygen.
 - Order 1. *Rhodobacteriales*

- b. Non-photosynthetic organisms.
 - Order 2. *Eubacteriales*
 - Order 3. *Actinomycetales*

- II. Unicellular rod-shaped organisms, without rigid cell walls. Always creeping motility. Microcysts and fruiting bodies may be formed. Class 2. *Myxobacteriae*
One order *Myxobacteriales*
- III. Unicellular, spiral organisms without rigid cell walls. Motility by means of an elastic axial filament or modified fibrillar membrane. Class 3. *Spirochaetae*
Order 1. *Spirochaetales*

It is among the Eubacteriales and related groups that are found most of the organisms which will be considered in the following chapters. It may be worthwhile, therefore, to present a hypothetical tree with four main ascending branches in which the representatives of this order are arranged on a purely morphological basis (Fig. 3).

Starting from the hypothetical primitive coccus type, the first line leads through the micrococci to the sarcinae, culminating in the spore forming sarcinae. These forms may be either Gram-positive or Gram-negative. Motility occurs infrequently in members of this group, and spore formation has so far been reported only in *Sporosarcina ureae* (Koblmüller, 1934).

The second line consists of the polarly flagellated rods, starting with the pseudomonas type and leading through the vibrios to the spirilla. The representatives of this line are all Gram-negative with one possible exception. Spore formation is rare, being well established for only one species, *Sporovibrio desulfuricans* (Starkey, 1938).

The third line is morphologically highly diverse. It takes its origin in the streptococci, and passes through the lactic acid rods, the propionic acid and other corynebacteria, and the mycobacteria to the actinomycetes. All members of this line are Gram-positive and none form endospores. However, there are organisms of the *Mycobacterium-Proactinomyces* type which are flagellated (Topping, 1937).

The fourth line comprises all peritrichously flagellated rods. Both Gram-positive and Gram-negative forms occur. It is here

that the great majority of spore formers have up until now been placed. This line is unsatisfactory because, particularly in the spore forming representatives, a number of morphologically distinct types can be recognized.

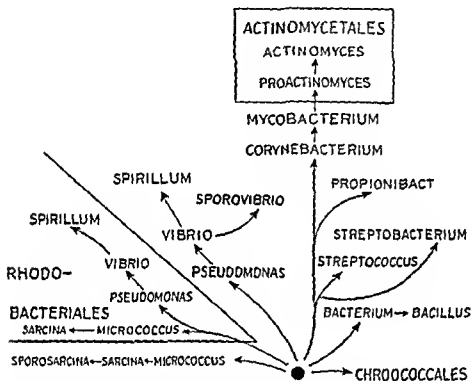


FIG. 3—Phylogeny of the *Eubacteriales* and related groups, according to the scheme of Kluver and van Niel with slight modifications. The names used denote morphological entities, not necessarily genera. For example, the designation "Bacterium" includes all genera of non-sporeforming, peritrichously flagellated rods *Kurthia*, *Escherichia*, *Aerobacter*, etc. (From Stanier and van Niel, 1941, fig. 1, p. 446.)

The above mentioned four lines were postulated primarily on the basis of cell shape and mode of insertion of flagella. It is of importance to note that the two most satisfactory groups (the first and the second) share an additional character, viz., the homogeneous behavior of the members with respect to the Gram stain.

In the remaining two lines no such uniformity exists at the present time. This makes it necessary to determine the relative values of the type of flagellation and of the Gram reaction for systematic purposes. The recent reports on the difficulties encountered in definitely ascertaining the mode of insertion of flagella lead one to suspect that the Gram stain may ultimately appear to be the more valuable (Pijper, 1930, 1931, 1938, 1940; Pietschmann, 1939; Conn, Wolfe, and Ford, 1940).

It is, of course, always dangerous and often unjustified to attempt any reconstruction of the trends and direction of evolution in any group of living beings. In the case of bacteria, the problem is further complicated, not only by the paucity of morphological characters available for classification, but furthermore by the great plasticity of this group of organisms. Not only can the cells of a single strain change their morphology depending upon the composition of the medium in which they are growing, but even under the same environmental conditions, coccoid, bacillary, and even filamentous forms are the normal expression of growth according to the "phase" (mucoid, smooth, rough, etc.) in which the species under consideration happens to be. Important cellular components such as spores, flagella, capsules, appear and disappear under conditions not always predictable, and there occur not only profound modifications of cellular and colonial morphology, but at the same time startling changes in biological behavior. The ability to produce certain enzymes, to synthesize pigments, amino acids, growth factors, etc., can be reversibly gained and lost by a given culture, even though derived from a single cell. Even more extraordinary is the fact that the faculty of producing certain polysaccharides, heretofore thought to be specific for each pneumococcus type, can experimentally be transferred from one type to another. This newly acquired property is then retained as a permanently transmissible factor (Chapter V).

Finally, it should be emphasized that, although most authors regard bacteria as primitive forms from which higher types have arisen, there are others who look upon them as having been derived from the higher forms by a retrograde evolution, *i.e.*, by

degradation or loss of certain characters. Thus it was recognized very early that the bacteria exhibit close relationship to the blue-green algae, but it is not clear whether the algae evolved from the bacteria or whether the latter are products of retrograde evolution. A *Chroococcus* sp., for example, would be indistinguishable from a *Micrococcus* sp. if it were to lose the capacity to produce photosynthetic pigments; the genus *Encapsis* would be similarly indistinguishable from the genus *Sarcina* as a result of similar modification. As we shall see, permanent loss of a structure or function is extremely common among bacteria and perhaps occurs as readily in the related blue-green algae.

In fact, the view that, instead of being the most primitive organisms, bacteria have evolved from higher forms of life by loss of structure and function, is repeatedly expressed in all fields of bacteriology. It is now suggested, for instance, that a long period of chemical synthesis of organic material preceded the emergence of life and that consequently the earliest living forms were heterotrophs. According to this reasoning, the autotrophic organisms, far from being original representations of life on earth, are the product of later adaptation to an environment in which organic materials had become scarce through the activity of heterotrophs (Oparin, 1938; Stanier and van Niel, 1941; van Niel, 1943). It has been stated that the Gram-negative intestinal bacilli, which exhibit such vigorous and varied fermentation reactions, "differ from one another by failure to ferment, or to ferment fully, one or more of a series of carbohydrates and other substances. Indeed, the whole trend of fermentation, in the parasitic group of intestinal bacteria, has been towards suppression." In the case of the typhoid bacillus, which requires tryptophane for its initial growth upon isolation from pathological material, but can grow in its absence after repeated transfers in artificial media, this changed requirement is thought to be due to the temporary loss of ability to synthesize the amino acid as a result of parasitic life in the human body. In fact, loss of ability to synthesize amino acids or other growth factors is apparently a common occurrence and accounts in part for the specific growth requirements of individual

microbial strains (Knight, 1936; Lwoff, 1932). We shall see that, although the loss of morphologic or antigenic constituents is often a reversible phenomenon, there are many cases where the variant strain appears to be stabilized in its "degraded" form; thus, flagellated or sporulated species may give rise to strains permanently devoid of flagella or spores. The multiplicity of salmonella types has been explained on the assumption that the simpler existing types were derived by loss variation from ancestors—probably related to *E. coli*—which were more complex both in fermentative power and antigenic structure (Edwards and Brunner, 1942; White, 1926, 1929). Even some of the filterable viruses, the macromolecules which appear today as the boundary between the living and the non-living world, have been regarded as the ultimate phase of retrograde evolution of higher cells. According to this view, complete adaptation to parasitic intracellular life has caused certain viruses to lose most of their vegetative faculties; while retaining the property of multiplication, they depend upon the host cell for the performance of synthetic processes.

3. DIRECT AND INDIRECT METHODS FOR THE STUDY OF CELLULAR STRUCTURE

The anatomical localization is often revealed first through the analysis of the physiological process.

CLAUDE BERNARD

Whatever the phylogeny of bacteria, it is certain that they constitute an extremely heterogeneous group of organisms and that their cells exhibit a great variability affecting, not only their physiological activities, but also their chemical composition and morphology. One may question, therefore, the value of any discussion dealing with the organization of the bacterial cell in general. It is true that individual studies of the various microbial species have revealed essential differences in the composition and properties of the respective cellular components. Progress in this line of work will undoubtedly contribute to the knowledge of phylogeny and will eventually permit generalizations and deductions concerning the characteristic cellular organization of each

bacterial group. The present survey is more limited in its scope. It is concerned primarily with those aspects of the problem which directly or indirectly have a bearing on the phenomena of infection. What are the laws which govern the extraordinary variability of the bacterial cell, according to which it adapts itself to the utilization of a new substrate, to the tolerance of an inimical environment, to the invasion of a new host? Are the hereditary characters in microbic life transmitted through the same type of nuclear apparatus utilized in higher organisms, or through other mechanisms as yet unrecognized and perhaps also present in other forms of life? What are the attributes which determine the property of virulence; can they be identified with cellular structures capable of protecting the pathogen against the defense of the host, or with physiological properties resulting in invasive power? (Chapter VI) Are the protective agents which Nature, and the physician, use in combating the infection, directed against the microbe as a whole or rather against certain of its most vulnerable structures? (Chapter VII) These questions and many others of obvious interest to the student of infectious diseases, would find a readier answer if it were possible to visualize the structural components of the bacterial cell, and to identify the nature of their reactions with the environment. The classical methods of cytology have contributed a great deal to this subject. By revealing the existence of spores, flagella, cell walls, and capsules, they have greatly aided in the understanding of many problems of epidemiology and immunity. Little by little, empirical staining reactions are gaining the dignity of cytochemical tests; with the help of monochromatic photography, especially in the ultraviolet range, they are giving chemical definition to morphological entities, allowing us to recognize possible nuclear structures among other cell granules. The electron microscope has greatly increased the resolving power of microscopy. Because of its lack of specificity it has in most cases given to the bacteriologist only a confirmation of classical knowledge, but one can expect that it will in the near future render visible new cellular elements, especially if used in conjunction with specific reagents.

These cytological techniques aim at a direct visualization of the cell, but there are other indirect methods of study which, by an analysis of the response of the cell to the effect of certain reagents and procedures, suggest the existence—and often the chemical nature—of important cellular components. As will be illustrated at some length in the following chapters, much of this indirect approach consists in the study of the reaction of the cell to immune antibodies, antiseptics and chemotherapeutic agents. It was Paul Ehrlich who first saw the possibility of describing these reactions in terms of cellular structures. He felt that, far from belonging to different disciplines, the laws of immunity and chemotherapy could be formulated in the same general terms. The living cell was assumed to possess a number of chemically reactive groups, called "receptors," with which dyes, bactericidal substances, and immune bodies reacted selectively. Ehrlich regarded these "receptors" as definite chemical entities, capable of entering into union with dyes, antiseptics and antibodies. Characteristic staining reactions, differential susceptibilities to toxic substances, and specific reactions with immune bodies could all be explained by postulating the existence of a sufficient number of receptors in the bacterial cell (Ehrlich, 1898, 1908, 1907). Unfortunately, neither Paul Ehrlich nor his immediate followers succeeded in identifying the chemical nature of these "receptors" or even in demonstrating their existence as well defined entities. During the past three decades, however, immunochemists, and students of the theory of chemotherapy, have gone far toward recognizing the nature, and in some cases separating in purified state, some of the cellular components with which antibodies and antibacterial agents react selectively. Thus, the "receptors" postulated by Ehrlich have now been given experimental reality and chemical definition (Chapters III, IV, VIII).

Nor are antibodies and antiseptics the only reagents which can be used to recognize and identify the cellular "receptors." If it is found, for instance, that a given enzyme attacks the cells of a certain microbial species, causing some alteration of a characteristic cellular property, it can be surmised that the chemical sub-

strate which is susceptible to this enzyme is present in the cell under consideration and that it plays some part in the function altered by the enzyme. If, furthermore, the enzyme or the antibody can react with the living cell, there is some likelihood that the cellular substrate, which is susceptible to it, is situated near the periphery of the cell, since the large molecular dimensions of antibodies and enzymes probably do not permit them to penetrate the plasma membrane. One can thus not only recognize the presence of certain specific components of the cell, but by bold, even though admittedly dangerous, extrapolation, guess at the approximate position of these components in the architecture of the cell. It will be pointed out later that the parts of the cell situated at or near the surface are of special interest to the student of immunity, since they constitute the exposed, vulnerable structures of the parasites against which the antibacterial defense can be directed (Chapter VII). The future will undoubtedly reveal other even more subtle physiological and biochemical techniques for the study of morphological problems. There is already some suggestion, for instance, that certain enzymatic functions are definitely associated with fairly well defined situations—phosphatases being concentrated at the periphery of the cell, adenosine triphosphatase being closely related to myosin—and that the coupling of oxidations and reductions requires the morphological association of the enzymes involved.

The indirect approach to cytology, which we are considering, utilizes chemical and biological manifestations as indices and guides to the recognition and identification of morphological structures. Its shortcoming is that it depends entirely upon the interpretation of results, and not upon direct observation, to establish the place of these structures in cellular organization.

At the present stage, when the unknown components of the cell exceed the known in number, the indirect physiological approach may appear to be unreliable. It should be pointed out, however, that the history of science provides many examples of the fruitfulness of indirect methods, as witness nuclear physics. The mode of experimentation and the reasoning involved in following this

approach are parallel to those by which the vast body of structural knowledge has been built up in the accepted domain of organic chemistry. Thus, the concept of the hydroxyl group was arrived at from the study of reactions such as those which occur when two compounds of the same empirical composition—ethyl alcohol and dimethylether—are treated with metallic sodium or a halogen acid. The fact that, in these reactions, hydrogen can be released either in association with, or independent of oxygen, depending upon the compound and the reagent used, revealed that one of the six hydrogen atoms occupies a unique position in the ethyl alcohol molecule probably in association with the oxygen atom, whereas the oxygen atom in dimethyl ether is involved in linking the two methyl groups and does not exist as a hydroxyl group.

Even in the case of the tissues and cells of higher organisms, important discoveries concerning their cytology and morphology have depended upon the use of extremely indirect physiological approach. The existence of chromosomes and genes, for instance, was surmised from the analysis of the transmission of hereditary characters before they could be seen as definite morphological entities; again, the recognition that the nervous impulse can be transmitted at different velocities revealed something of the significance of the diameters of the different fibers within the same nerve bundle. In the words of Claude Bernard (1855-56) "anatomical localization is often revealed first through the analysis of the physiological process." Many illustrations of this view could be found in the past, but instead of describing them, it may be sufficient to mention a situation which bids fair, in the near future, to illustrate again the power of the method. It is well known that the analysis of the immune reactions elicited by the injection of erythrocytes into experimental animals has revealed the existence in these cells of substances heretofore unrecognized, and one may confidently expect that the isolation of the specific polysaccharide of blood group A, for instance, will result in a knowledge of its role and place in the cells from which it is derived. Similarly, an understanding of the mechanism of hemolysis

caused by specific anti-erythrocyte agents will help in describing the organization of the red cell. In brief, one may predict that immunological methods, as well as the study of the mode of action of enzymes and other reagents on different types of cells, will in the future become one of the techniques for the study of the morphological, chemical, and physiological aspects of cytology.

Much of the morphology indirectly revealed by antibodies, enzymes, and cytotoxic substances lies beyond the microscopic range, and does, in fact, often reach the molecular level. It concerns the organization of those molecular groupings which, on account of their chemical activity, condition the behavior of the cell both as an independent functioning unit and in its relation to the environment. The ultimate understanding of the natural history of infectious diseases, and the rational development of methods for their control, depend upon this knowledge. It is probable that, without it, epidemiology would have remained to a large extent a mere statistical statement of mysterious events; the pathology of infectious diseases a purely descriptive science; immunity and chemotherapy a set of empirical procedures.

II

CYTOLOGY OF BACTERIA

. . . so long as the makers of microscopes do not place at our disposal much higher powers, and, as far as possible, without immersion, we will find ourselves in the domain of the bacteria, in the situation of the traveller who wanders in an unknown country at the hour of twilight at the moment when the light of day no longer suffices to enable him clearly to distinguish objects, and when he is conscious that, notwithstanding all his precautions, he is liable to lose his way.

FERDINAND COHN

IN spite of their minute dimensions, bacteria do not constitute an undifferentiated mass of protoplasm. The bacterial body is a well organized cell consisting of a protoplast encased in a nonprotoplasmic cell wall; the surface of the protoplast is a cytoplasmic membrane, and there exist within the cytoplasm sap vacuoles and various nonprotoplasmic granules which are more or less readily identified by microchemical reactions. Other special structures—endospores, flagella, capsules, etc.—occur only in certain species or even in certain phases of the growth and life cycles of these species.

The shape, dimensions, and motion of bacteria, the mechanism of cell division, spore formation and spore germination, the nature of cellular inclusion bodies, etc., are subjects which are of interest to the cytologist and to the student of infectious diseases as well, because they affect the interpretation of many of the phenomena of infection. Fortunately, the description of the structure of the bacterial cell in terms of classical cytology has been presented in a number of books and reviews, and it will be sufficient to focus our attention on those aspects of the problem which appear to have a bearing on the infectious process (Benecke, 1912; Delaporte, 1939; Frost and Guilliermond, 1917; Hollande, 1934;

Knaysi, 1938, 1944; Gotschlich, 1929; Lewis, 1941; Meyer, 1912; St. John-Brooks, 1930).

1. LIMITATIONS OF MICROSCOPIC METHODS

Microscopy in the Visible Range.—Most bacterial cells do not exceed $1\ \mu$ in diameter, and some in fact are even smaller. The limit of resolution in visible light being approximately $0.15\ \mu$, it is obvious that only structures which occupy a fairly large percentage of the total cell volume are visible with ordinary microscopy. Failure to recognize this limitation of the optical methods has led some authors to describe cellular objects far too small to be seen, such as centrosomes, with dimensions of $0.01\ \mu$, and it is certain that many of the controversies and contradictions which characterize the literature dealing with the finer structure of bacteria are due to attempts to strain beyond reason the magnifying power of the microscope (Enderlein, 1925). On the other hand, it is possible that there exist in bacteria important cellular structures which have remained unrecognized because they are too small to be detected by ordinary microscopic methods.

Ultraviolet Photography.—Microscopy in the ultraviolet gives an increased resolving power due to the shorter wave length of the light used, and bacterial cells contain a number of structures which strongly absorb light in this range. Thus nucleic acid bodies are readily detected in the ultraviolet on account of their high content in purine and pyrimidine bases, substances which exhibit strong absorption in the region of $2650\ \text{\AA}$ units. Unfortunately, the technique of photography at different wave lengths is not sufficiently developed to allow the separation of many types of structures. It is possible, however, to introduce an element of specificity in ultraviolet photography by using it in conjunction with other techniques. Thus, by treating a preparation with the enzyme ribonuclease and comparing its behavior toward ultraviolet photography and staining before and after enzyme treatment, it can be shown that the basophilic character of the cytoplasm is due in large part to its ribonucleic acid content (Mirsky, 1943).

Definition of the characteristic absorption patterns of different chemical groups and improvements in the technique of photography with monochromatic lights will eventually increase the possibilities of microscopy in the ultraviolet, not only from the point of view of greater resolving power, but more especially towards a more accurate identification of the chemical groups which absorb light in this range (Caspersson, 1940; Caspersson and Thorell, 1942; Wyckoff and Ter Louw, 1931).

Electronic Microscopy.—Although the electron microscope permits enormous magnifications (up to 100,000 diameters), the necessity of operating *in vacuo* and therefore on completely desiccated material limits the type of biological observations possible with this instrument. Electronic microscopy also suffers from lack of chemical specificity since it detects only differences of opacity to high velocity electrons. The contrast of image in electron microscopy is due to the scattering of electrons, and the scattering is a function of both thickness and density of the specimen. It is fortunately possible to change the density by the addition of certain reagents capable of combining with the material in the preparation, and this permits improving the specificity of the methods by the use of reagents which combine more or less selectively with certain cellular components. These reagents take the place, in other words, of the stains used in visible microscopy, and present over them the advantage of greater range of selection and of greater specificity of action, since they are not limited to substances possessing tinctorial properties (Marton, 1943; Morton and Anderson, 1941; Mudd and Anderson, 1941, 1942).

Intracellular and Extracellular Granules.—The presence in bacterial cultures of cells which are abnormal in morphology or dimensions, of such small size, for instance, that they can go through bacteriological filters, has been used as evidence of the existence of complex life cycles characterized by modes of reproduction other than simple fission. It must be kept in mind, however, that many physical and chemical properties of the environment, such as the surface tension of the medium, the abundance of oxygen supply, the concentration of nutrients and of elec-

trolytes, etc., greatly affect not only the shape and the dimensions of bacterial cells, but also the comparative rates at which protoplasm synthesis, cell division, and cellular disintegration take place. Staining reactions are not adequate for the differentiation between small vegetative cells and lifeless granules consisting of storage material or cellular breakdown products. Furthermore, the techniques required for the separation of these cellular elements and for the study of their viability and behavior are either not available or extremely cumbersome. It is clear, therefore, that

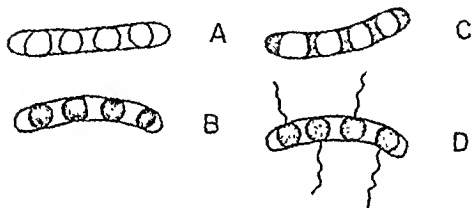


FIG. 4.—Diagrammatic drawings illustrating the appearance of unstained cell of *Rhizobium trifolii* and the same with different staining methods. A, unstained showing refractive granules. B, fat bodies stained by the naphthol blue method. The cytoplasm is colorless. C, stained with methylene blue. The unstained fat bodies alternate with stained bands of cytoplasm. D, flagellated banded cell according to Thornton and Gangulee (From Lewis, 1938, fig. 3, pl. 1, p. 587.)

the results derived from mere microscopic observation, or from filtrability experiments, require the most critical scrutiny before they can be used to identify as reproductive structures the various types of granules which may occur extracellularly or intracellularly in bacterial cultures (Chapter V: 6).

There has been a tendency to regard as nuclei or chromidia all the inclusion bodies which stain deeply with the basic, so called nuclear, dyes. It is true that desoxyribonucleic acid is an essential component of the nucleus of higher cells, and that this substance exhibits great affinity for the basic stains. It is also true, on the

other hand, that ribonucleic acid possesses the same staining characteristics, and is present in many bacterial cells as refractile bodies—variously designated as Babes-Ernst granules, metachromatic granules, volutin granules. These volutin granules are not permanent structures; their deposition depends upon the species, the age of the cell, and the composition of the medium. The fact that they disappear as a result of starvation suggests that they function as a storage-food product, and not as essential structural constituents in the microbial species in which they occur (Caspersson and Brandt, 1941; Delaporte, 1939; Grimme, 1902; Gröh, 1938; Lewis, 1941; Painter and Taylor, 1942).

Other refractive granules consist of glycogen or of some other ill-defined reserve polysaccharide which stains blue with iodine and which is designated as granulose or iogen. Fat bodies are also very common and exhibit a position within the cell which is characteristic for the organisms of a particular genus (Burdon, 1944; Burdon, Stokes, and Kimbrough, 1942). The occurrence of several fat bodies within a single cell often results in the compression and breaking up of the cytoplasm into several fragments which, because of their small size and intense staining, have at times been regarded as chromatin or reproductive bodies. In other cases, the fat bodies themselves have been taken for endospores because of their refractility (Lewis, 1938, 1941, 1942).

2. PROBLEMS OF THE NUCLEUS

Criteria of the Existence of a Nucleus.—It has been assumed that bacteria, standing at the threshold of organized living matter, are devoid of any structure suggesting a nucleus, and are therefore representatives of those cell-like organisms which, because they are not nucleated, have been called cytodes by Haeckel. During the past few decades, however, attempts have been made to analyze, in terms of classical genetics, the process of transmission of hereditary factors in bacteria, and the mutation-like phenomena which they exhibit so frequently. Many have taken for granted that these phenomena occur through the agency of genes

organized as chromosomes in a nucleus, similar to the structure found in higher cells. The existence of true nuclei has indeed been convincingly demonstrated in all other groups of microorganisms with the single exception of the blue green algae, organisms which, interestingly enough, happen to be closely allied by phylogeny to several groups of bacteria. It must be pointed out, in this respect, that blue green algae possess a structure—the central body—which is rich in desoxyribonucleic acid, and which has been considered as a primitive nucleus sharply differentiated from the protoplasm.

All the early claims of the existence of nuclei in bacteria rested upon the result of staining reactions due to the existence in these cells of certain substances—either diffuse in distribution or organized in the form of granules—which react toward the basic aniline dyes like the chromatin of higher organisms. Interpretations of these microscopic observations have ranged all the way from the hypothesis that bacteria are not nucleated, to the view that the entire bacterial cell is a nucleus, the cytoplasm being lacking or reduced to an invisible layer. It would not be profitable to review in detail the many conflicting descriptions and theories dealing with this problem; it appears sufficient to summarize them by quoting directly from a recent critical review in which the different theories are divided into the following groups.

1. The bacteria do not possess a nucleus or its equivalent.
2. The cell is differentiated into a chromatin-containing central body and peripheral cytoplasm.
3. The bacterial body is a nucleus devoid of cytoplasm: a naked nucleus or nuclear cell.
4. The nucleus consists of several chromatin bodies, a chromidial system, scattered throughout the cytoplasm.
5. The form of the nucleus is not constant throughout the growth cycle; it may occur as a discrete spherical body, an elongated chromatin thread, or scattered chromidia depending on the stage of development: a polymorphic nucleus.
6. The nuclear substance consists of fine particles of chromatin dispersed uniformly in the cytoplasm but is not distinguishable as morphological units: a diffuse nucleus.

7. The protoplast contains one or more true vesicular nuclei.
8. The nucleus is a naked invisible gene string, or a chromatin-encrusted gene string analogous to a single chromosome. (Lewis, 1941)

The fact that these hypotheses encompass all the possible solutions of the problem indicate some fundamental difficulty in its analysis. Of obvious importance in this respect is the minute dimension of bacteria which brings any intracellular body close to, or even below, the limit of resolution of visual microscopy. Even more important, perhaps, is the lack of adequate criteria to define the nucleus and to test for its presence.

In higher organisms, the nucleus is a structure morphologically distinct from the cytoplasm, consisting largely of nucleoprotein (the nucleic acid being of the desoxyribose type), undergoing a complex and characteristic process of division associated with cellular division, and acting as the bearer of the hereditary characters. Neither the staining reactions, nor the process of nuclear division, nor the analysis of the transmissibility of hereditary characters can be used as a convincing criterion of the existence of a nucleus in the bacterial cell. As already stated, many bacteria contain a number of intracellular granules of acidic character which exhibit great affinity for the basic (so called nuclear) dyes; it is certain in particular that so called volutin granules (which probably consist of reserve ribonucleic acid), and which stain very deeply with basic dyes, have often been taken for nuclei. On the other hand, the mere fact that a basophilic intracellular body is seen to undergo division is not evidence of its nuclear nature. There are other cellular structures, such as the mitochondria and the chloroplasts which also divide, and nothing even remotely resembling the process of karyokinesis has so far been demonstrated in bacteria. Finally, it has not yet been proven that the transmission of hereditary characters in these organisms obeys the mendelian laws and it is not possible therefore to argue from a similarity of biological behavior to an identity of cellular structure. Granted the absence of convincing evidence, there have nevertheless been indications of the existence in bacteria of cellular bodies which possess some of the properties of a true nucleus.

Results of Ultraviolet Microscopy.—On the assumption that all nuclei are very rich in nucleic acid, much of the modern work has been concerned with attempts to demonstrate the presence in the bacterial cell of structures containing this type of substance. To this end, bacteria have been photographed in ultraviolet light in order to take advantage of the strong absorption exhibited by purine and pyrimidine bases in the region of 2650 Å, with results as conflicting as those obtained by the use of basic dyes.

Some observers have seen in the aerobic sporulating bacilli, in staphylococci, in sarcina, and in the Gram-negative bacilli, images which suggest that bacteria contain a nucleus which undergoes mitotic division, whereas others, also using *B. subtilis*, could not recognize any nuclear structure (Barnard, 1930; Piekarski, 1938; Wyckoff and Ter Louw, 1931). Absorption in the 2650 Å range is due chiefly to the purine and pyrimidine bases. Since, in addition to being present in true nuclei in the form of desoxyribonucleic acid, these substances are also present in many important protoplasmic constituents (coenzymes, ribonucleic acid, etc.), it is obvious that ultraviolet photography is not much more specific for the purpose of demonstrating a nucleus than are the old basic nuclear dyes.

The limitation of ultraviolet photography in this respect becomes even more serious when it is realized that bacterial cells are extremely rich in nucleic acid. In hemolytic streptococci, for example, over twenty per cent of the total cell weight consists of this group of substances, the largest part being of the ribose type (Sevag, Smolens, and Lackman, 1940). An additional difficulty arises from the fact that the two different nucleic acids, long thought to be unrelated, seem on the contrary to be in a constant state of metabolic inter-relationship, the desoxyribonucleic acid present in the nucleus being produced by reduction of the ribo compound which is more abundant in the cytoplasm (Koller, 1943; Mirsky, 1943; Mitchell, 1942).

Results of Electronic Microscopy.—Several attempts have been made to establish the existence of a nucleus by studying bacteria with the electronic microscope. It has thus been confirmed that

certain bacterial species contain intracellular bodies which often appear paired or constricted, indicating a probable division. Unfortunately, the technique has not afforded any greater specificity, or any better picture of the intimate nature of the division process (Knaysi and Mudd, 1943; Piekarski and Ruska, 1939).

The granules revealed by electronic microscopy appear to be much smaller, or even not visible at all, when very young cells are examined. Furthermore, although certain strains of staphylococci and meningococci show intracellular granules, other cultures of staphylococci, streptococci, and gonococci show no internal structure even when the voltage used is high enough to render the cells transparent. In order to account for these inconsistencies, it has been suggested that the state of the nuclear material changes not only from one strain to another, but also during the growth cycle of a single culture. If this interpretation is the correct one, the old views according to which the nucleus exhibits a diffuse distribution, or possesses a granular structure, or exists as a central body, are not contradictory, but describe in reality the state of the nuclear material at a given time for a given phase of the culture (Knaysi and Mudd, 1943).

Results Obtained with the Feulgen Technique.—Since the nuclei of higher cells contain desoxyribonucleic acid as one of their essential constituents, and since the Feulgen reaction, when used under proper conditions, serves as a fairly specific color test for this substance, many authors have attempted to demonstrate in bacteria intracellular bodies staining positively by the Feulgen technique. Although all bacteria give indeed a positive nuclear reaction after treatment with hydrochloric acid, it should be mentioned that some workers still question the specificity of the Feulgen reaction, and consider that the material which it stains is not necessarily true chromatin or genes, but can be in the nature of food reserve (Knaysi, 1938; Margolena, 1932; Sander, 1938; Schaede, 1939).

The opinion prevailed until recently that the Feulgen positive substance occurs normally in the bacterial cell in the form of very

minute particles uniformly dispersed throughout the cytoplasm. It has now been shown, however, that the diffuse condition is often an artefact caused by the high temperature of hydrolysis. Acid treatment at or above 60° C. not only liberates the desoxyribo sugar, but causes at the same time a destruction of the cellular structure and thus abolishes any localization within it. When, on the other hand, hydrolysis is carefully controlled, the Feulgen positive substance is found to be present in fairly well defined bodies, whereas the rest of the cytoplasm remains unstained (Stille, 1937).

Careful utilization of the nucleal reaction has convinced many authors that bacteria contain well defined Feulgen positive structures which divide before cellular division, which react like chromatin with the basic nuclear dyes, and which are comparable to the chromosomes of plant and animal cells. Similar results have been obtained with a great number of well identified bacterial species, including staphylococci, sporulating bacilli, Gram-negative bacilli, and the regularity in form and position of the Feulgen positive bodies rules out the possibility of artefacts. These structures have been termed nucleoids, chromatinic bodies, or ebromosomes. They occur in spores as well as in vegetative forms, and are believed to contain the hereditary mechanism of the bacterial cell (Badian, 1933; Delaporte, 1939; Dienes and Smith, 1943; Knaysi, 1942; Lewis, 1942; Milovidov, 1935; Neumann, 1941; Pickarski, 1937, 1938; Pietschmann and Rippel, 1932; Robinow, 1942, 1944; Stille, 1937).

It is of some interest to point out at this time that, whereas ribonucleic acid can be extracted readily from pneumococci and from yeast by mild enzymatic action and by neutral solvents, desoxyribonucleic acid is much more firmly bound with the cell structure from which it can be released only by cytolysis or by strong alkali (Avery, MacLeod, and McCarty, 1944; Delaporte, 1939, p. 449; Thompson and Dubos, 1938).

One specific example will serve to summarize the type of cytological evidence which is offered to demonstrate the presence of

a nucleus in bacteria. A strain of staphylococcus was found to contain an intracellular body exhibiting metachromatic staining with old solutions of methylene blue. The material constituting this body did not disappear on starvation of the culture and was of such strong acid character that it stained deeply with methylene blue at pH 1.8 to 2.0. It was not dissolved by boiling 0.5 per cent bicarbonate solution and gave a clear Feulgen reaction after controlled acid hydrolysis. All these properties are compatible with the conclusion that the material in question consists essentially of desoxyribonucleic acid. This intracellular body, although occupying a large proportion of the cell, could not be seen in the living state, indicating that its refractive index was close to that of the cytoplasm. It was considered to correspond to the nucleus of higher cells (Knaysi, 1942).

Granted that the transmission of hereditary characters in bacteria presents at least some analogy with the same process as it occurs in higher cells, one may assume that this process takes place through the agency of genes. These genes then should maintain a fixed position in regard to each other; they should synchronize in division and be distributed in such a manner that a full complement of them could find its way into each daughter cell. A nucleus, reduced to the lowest essentials necessary to meet these requirements, could consist of a single gene string existing as a small granule or as a rod-like body rather than as a definite vesicle separating it from the cytoplasm (Allen, Appleby, and Wolf, 1939; Badian, 1933; Lewis, 1940; Lindegren, 1935; Lindegren and Mellon, 1932).

Although there exists some evidence for the view that the nucleus of bacteria consists of a single gene string, this hypothesis has probably become unnecessary in the light of the new information concerning the chromosome-like bodies which have been described in the preceding pages. Adaptation of the classical staining reactions to the study of bacterial cytology has provided such striking pictures of several morphological structures of the bacterial cell that one may expect great progress from these techniques in the near future (Robinow, 1942, 1944, and Addendum).

3. ENDOSPORES

The endospores of bacteria appear to be produced by a condensation of the cytoplasm of the vegetative cell and are liberated by cellular disintegration. Although there are isolated observations of the existence of two spores within one sporangium, it appears that endospores are not reproductive in function, but rather constitute resistant, resting forms. This view is substantiated by the fact that sporulation is greatly enhanced by exhaustion of the nutrients in the medium (Cook, 1932; Knaysi, 1938; Migula, 1904).

Some workers believe that endospores may be formed by the aggregation of a number of chromophilic granules which migrate to one of the poles of the cell (Knaysi, 1938). It is more generally accepted, however, that the spore is formed from a clear hyaline polar spore primordium which is set off from the remainder of the cell by a membrane, the spore itself resulting from a condensation of the substance enclosed in the spore primordium (Knaysi, 1938; Lewis, 1941). The spore body stains readily with aniline dyes before it has become fully condensed. As the spore matures, however, it becomes refractile and no longer stains except as a result of drastic treatment, involving for instance the use of carbol fuchsin and heat. The mature spore is sharply separated from the cytoplasm by a spore wall; in fact, the spore coat is claimed to consist of three layers, an intine, an exine, and an outermost part, the slimy layer. Germination of the spore begins by simultaneous swelling and loss in refrangibility, followed by a cracking or a digestion of the exine (Knaysi, 1938).

The material which condenses to form the spore apparently consists in large part of chromatin-like, nuclear material. Indeed, some observers claim that at the time of sporulation, the nucleus, consisting of a single chromosome, divides lengthwise equally, the two chromosomes subsequently uniting in an autogamous sexual process accompanied by chromatin reduction. There are also reports of division in four haploid chromosomes, of which three

eventually break down, the fourth one giving rise to the spore (Badian, 1933; Schaudinn, 1902, 1903). On the other hand, other workers failed to observe any fusion preceding spore formation and claim that the vegetative cell which is ready to form a spore contains two Feulgen positive bodies, only one of which participates in spore formation, the other being lost with the death of the mother cell (Stille, 1937). On the whole it does not appear that autogamy has been convincingly proven to occur during spore formation.

The failure of the mature spore to stain by ordinary techniques is often ascribed to the relative impermeability of the spore wall. When, however, free spores are treated by ordinary staining methods, it often happens that the spore wall stains deeply, while the interior remains uncolored, a fact which suggests that bacterial protoplasm undergoes some profound modification of its physico-chemical character as a result of sporulation. When sporulating forms are treated with Wright's stain at pH 7.6, the spores are stained deep blue while the cytoplasm of the sporangium is stained pink red. Spores stained by this method appear smaller than when stained by the usual techniques; this fact has been interpreted to mean that the spore wall is left unstained by this technique, a view compatible with the observation that free spores resuspended in nigrosin are almost always surrounded by a narrow colorless zone (Dutton, 1928).

Although both the spores and vegetative cells of *Bacillus mycoides* give the same analysis for ash, moisture, and protein, the spores lack enzymic activity. In order to account for this situation, it has been assumed that the enzymes are combined in some obscure way by their active groups and thus become at the same time inactive and heat resistant (Virtanen and Pulkki, 1933). Contrary to expectation, the total water content of the vegetative cells and of the spores is the same. Cryoscopic analysis has revealed, on the other hand, that the vegetative cells contain a much greater proportion of their total water as free, unbound water. It has been suggested that the heat resistance of spores is due to the fact that the water which they contain, being in the

bound form, cannot take part in heat coagulation of the proteins (a questionable concept) (Friedman and Henry, 1938).

The spectrochemical analysis of twelve aerobic sporulating species has revealed that the spores are materially higher in calcium and manganese, and lower in potassium and phosphorus than the vegetative cells from which they are derived. In general, high concentrations of calcium are associated with enhanced heat resistance, a fact perhaps correlated with the greater water binding capacity of spores (Curran, Brunstetter, and Myers, 1943). Attempts have also been made to account for the resistance of spores in terms of their contents of fatty substances. Extraction with benzene, acetone, amyl alcohol, and carbon tetrachloride does not, however, modify the resistance, which is reduced only by prolonged extraction with chloralhydrate and trichlorethylene (v. Angerer, 1938). The heat resistance of the spore appears to be related to the toughness of its exine, since species which shed their exine upon germination of the spore are more resistant to heat than those which absorb it (Knaysi, 1938).

Spore production is conditioned by a number of ill defined factors, such as exhaustion of nutrients, aeration, presence of calcium, etc. (Bordet and Renaux, 1930; Cook, 1932; Greene, 1938; Kaplan and Williams, 1941). Sporulating strains, even issued from single cells, often give rise to daughter cultures which lose—in many cases permanently—the ability to produce spores. On the other hand, the property of sporulation, long considered characteristic of the rod-shaped bacteria of the family Bacillaceae, has now been recognized among cocci and vibrios (Gibson, 1935; Starkey, 1938). The fact that *Vibrio desulfuricans* forms spores only when grown at 45° to 55° C. but not below 40° C., illustrates that much remains to be learned, not only of the mode of formation and of the physicochemical and biological properties of these resistant forms, but also of their occurrence in the bacterial world.

The extraordinary resistance of endospores to heat and to all sorts of toxic agents gives them a unique position in the world of living things, and was responsible for prolonging the controversy

on spontaneous generation. It also accounts for their great practical importance in the problems of epidemiology. Finally, the fact that enzymes and other biological systems exist in an inactive and resistant form in these structures, and become again active and susceptible during the obscure phenomena of spore germination, presents to the physiologist and the biochemist problems of great originality and importance.

4. CELL ENVELOPES

The structures which surround the bacterial cell have been indiscriminately described under the names membrane, wall, cortex, rindenschicht, ectoplasm, capsule, slimy layer, sheath, etc., and these words have been employed by different authors to mean different structures, to account for unspecific staining reactions, and for observations of physicochemical properties. The confusion in terminology has been increased still further by the immunologists who, on the basis of serological and other immunological evidence, have claimed that certain substances extracted from bacteria belong to the cell surface, without specifying whether the surface in question refers to the cytoplasmic membrane, to the rigid wall which encases the cytoplasmic constituents, or to the indefinite layer of excreted products which accumulates around the cell proper. At the risk of being dogmatic, the information derived from staining reactions, and from the physicochemical and biochemical properties and behavior of bacteria, will be described in the present chapter according to the pattern commonly employed in the description of animal and plant cells.

The Cytoplasmic Membrane.—Like other cells, bacteria can undergo plasmolysis when placed in fluids of osmotic pressure higher than that of the protoplasm, and the cytoplasm can be shown to shrink away from the cell wall under these conditions. In fact, the ability to plasmolyze is retained by certain strains of sporulating bacteria even after the process has been repeated up to eight times (Fischer, 1897; Imsenecki, 1937). The ease

with which bacteria can be plasmolyzed varies from one species to another, with the nature of the medium in which they are growing, and with the age of the cell; young organisms are in particular much more susceptible to plasmolysis than the older ones (Sherman and Albus, 1923).

Although bacteria can in general survive much more readily than other organisms in distilled water, the effect of osmotic pressure often expresses itself in the phenomenon of plasmoptysis, in which the protoplasm is extruded at one end where it collects as a spherical mass. The reaction can be brought about by suddenly transferring growing cells from a highly osmotic medium into distilled water (Raichel, 1928; Sherman and Cameron, 1934). When luminescent marine bacteria (*Achromobacter harveyi*) are placed in distilled water, luminescence and motility cease immediately. Cytolysis occurs although the cells do not swell, but rupture and crack instead, the refractive index of the ruptured bacterial ghost becoming approximately that of water. The normal osmotic pressure of these organisms appears to reach up to 22 atmospheres (Johnson and Harvey, 1937; Johnson, Zworykin, and Warren, 1943).

Although bacteria behave as osmotic systems and possess a semipermeable membrane, only few quantitative studies have been made of the property of permeability. Comparison of the behavior of an alga (*Chara*), and of a coliform bacillus is of some interest in this respect. When the alga was placed in a glycerine solution, it took forty-eight hours before the glycerine concentration in the interior of the cell could reach one-half of the concentration in the external solution, whereas the same result was achieved within one minute by the coliform organism. The conclusion that the permeability properties were either qualitatively different in nature, or at least of a different order of magnitude, would, however, be erroneous, since only a very slight difference between the two types of cells could be recognized when permeability was expressed in terms of unit cell area (Collander, 1937).

It is likely that more quantitative information concerning the

properties of semipermeability would greatly help in the analysis of many problems of bacterial metabolism, such as the mechanism by which bacterial species, and especially mycobacteria, can maintain their internal environment within very broad zones of acidity and basicity (Cohen and Clark, 1919; Loebel, Shorr, and Richardson, 1930; Richardson, Shorr, and Loebel, 1931). The fact that the pH optimum of several enzymes appears to be very different when intact living bacteria and soluble preparations extracted from them are used as source of enzymes, suggests that the intracellular pH is to some extent independent of the environment (Chapter IV: 1).

The nature and dimensions of the cytoplasmic membrane which is assumed to be responsible for the properties of semipermeability is largely a matter of conjecture. It is obvious that some of the protoplasmic constituents must become concentrated at the surface of contact of the cytoplasm and environment, and form there a membrane consisting essentially in a dense layer of protoplasm. Direct microscopic observations have convinced some authors that this cytoplasmic membrane starts in the young cell as an interfacial fluid film and gets thicker and denser as surface active material accumulates, finally becoming a firm structure made up sometimes of several layers. This membrane is drawn in with the cytoplasm when the cell is plasmolyzed. It exhibits very strong affinity for dyes and is one of the last structures to be decolorized in the Gram and Ziehl-Neelsen staining techniques (Knaysi, 1930). This membrane, which is hyperchromatic and brilliant in the darkfield, has been assumed to consist of lipids and lipoproteins (Knaysi, 1929).

That such a cytoplasmic membrane exists is therefore well established on morphological grounds. It is less certain, however, that the physiologically active part of the membrane, that which is responsible for the properties of semipermeability, is thick enough to be detected by ordinary microscopic technique. In yeast, analysis of impedance of cell suspensions indicates that the membrane has a thickness of the order of 0.005μ (Fricke and Curtis, 1934); in mammalian erythrocytes, a study of the optical

properties and of lysis by certain agents suggests also that the membrane is a protein-lipid complex of a thickness of 0.003μ (Harvey and Danielli, 1938; Ponder, 1937; Schulman, 1937). One may assume that the semipermeable membrane of bacteria possesses similar properties, although adequate observations and measurements are not yet available to settle the point.

The Cell Wall.—We have seen that the cytoplasm of the bacterial cell lies freely within a cell wall from which it can be separated by plasmolysis. In the fully turgid cell, the cytoplasmic membrane is in close contact with the outer wall and is not readily differentiated from it. In hypertonic solutions, on the contrary, the cytoplasmic membrane is drawn in with the contracted cytoplasm while the rigid outer wall retains its shape. As already mentioned, the existence of a rigid cell wall in *Achromobacter harveyi* can be readily demonstrated by resuspending these organisms in distilled water (fig. 1). It has been shown by staining and darkfield observation that, in this case at least, the cell wall is able to fix calcium and magnesium. Electron microscopy has revealed that the lysed cell ghosts of these marine bacteria exhibit a mosaic structure with relatively transparent roughly circular or slit-like areas, the smallest of which are $5 m\mu$ in diameter (Johnson and Harvey, 1937; Johnson, Zworykin and Warren, 1943). In the case of pneumococci, it is also possible to differentiate by electron microscopy the cell wall from the capsule which surrounds it, and from the inner protoplasm which is limited by a cytoplasmic membrane (Mudd, Heinmets, and Anderson, 1943).

In addition to the evidence afforded by direct microscopic observation, there are other facts which establish the existence of a rigid cell wall in many species of bacteria. The most obvious perhaps is the very rigidity of the cell and the maintenance of a constant cell form other than spherical. A naked mass of protoplasm resuspended in water would become spherical due to the surface action acting upon it; but the majority of bacterial cells are elongated, straight, or curved rods, and behave as if the protoplasm were encased within rigid tubes. The rigidity of the cell wall can

be demonstrated directly by microdissection experiments, and also by the fact that if the cell in its motion meets an obstruction it acts like a solid rigid body; it may bend but later resumes its former shape (Wámoscher, 1930).

Some aspects of bacterial structure can also be revealed by the use of oils of different refractive indices as suspension medium. Under these conditions of observation, and in the case of organisms as different as anthrax, diphtheria, and proteus bacilli, it is possible *in vivo* to blot out the cell wall and to reveal internal granules or *vice versa*. When streptococci and staphylococci are observed in media in which the cell wall is visible, their total diameter appears larger by $0.5\ \mu$ than when the cells are studied under other conditions (Eisenberg, 1930). The cell wall and intracellular granules can also be made visible by illuminating the preparation at different azimuths, thus permitting observation in natural media (Eisenberg, 1932).

In general, ordinary or darkfield illumination is not sufficient to reveal clearly the cell wall, and more satisfactory pictures have been obtained by microscopy in the ultraviolet or with the electronic microscope (figs. 1 and 2). Although the wall exhibits only little affinity for dyes, and, for instance, does not react with methylene blue, it can be colored yellow with safranin and purple with methyl violet. In fact, when *Bacillus subtilis* is stained *intra vitam* with dilute solutions of crystal violet, there is seen a purple outer wall surrounding a dark violet protoplasmic membrane which encloses the cytoplasm, while the latter is of a much lighter shade (Knaysi, 1930). The cell wall can be demonstrated even more clearly when the staining reaction is carried out on bacteria which have been plasmolyzed by mounting them in 25 per cent NaCl. Other more complex staining reactions which attempt to differentiate between the walls of Gram-positive and Gram-negative organisms have also been devised and will be considered again in Chapter III: 3 (Gutstein, 1925; Maneval, 1929).

Unfortunately, very little information is available concerning the chemical composition of the wall, and the many claims that it consists of cellulose, or of chitin, have not been substantiated

(van Wisselingh, 1925). Lack of affinity for dyes and great resistance to chemical treatment (except to strong acids and alkalis) suggest that it consists of a non-reactive substance and that its function is chiefly concerned with mechanical protection of the cell constituents. As will be shown in Chapters III and IV, the analysis of the behavior toward dyes, antibodies, and enzymes is providing much useful information concerning the properties and the chemical nature of the cell wall.

The Capsule.—Many bacterial species produce, under the proper circumstances, high molecular mucoid substances which diffuse into the medium. When this viscous material remains concentrated around the cell, it constitutes a structure which has been called capsule, sheath, or slime layer, and which can be demonstrated by adequate techniques of positive or negative staining. The presence of the capsular material usually gives to the culture which produces it a stringy texture, and to the colonies on solid media a moist, glistening surface which is qualified as mucoid. Capsule production is not a strain characteristic; it depends in part upon the conditions under which the culture is growing and more especially upon the dissociation phase in which the organism happens to be. The property to produce a capsule, when lost, is readily regained by some strains, and with difficulty or not at all by others. In any case, it must be emphasized that far from being a rare property, capsule production has been recognized in practically all bacterial species, saprophytic as well as pathogenic. It is of some interest in this respect that capsules are a very characteristic feature of nearly all algae, and that in some of the unicellular representatives of this group (which appear to be phylogenetically related to certain bacteria), they present the same general appearance as do the homologous structures in bacteria, and like most of them consist of complex polysaccharides.

Although the production of capsular material is a hereditary property which is lost only when the culture undergoes phase variation, there are environmental factors which appear to influence it directly. Thus, capsule formation is stimulated when the anthrax bacillus is grown in animal tissues, and when organisms of

the colon-typhoid group are cultivated at low temperature (10° to 20° C.) in the presence of fermentable carbohydrates (Preis, 1911; Morgan and Beckwith, 1939). So little is known of the influence of the environment on capsule production that it cannot be analyzed at the present time. In some cases, the medium probably favors the selection of a normally occurring capsule-producing mutant. In other cases, the presence of certain nutrients may be required for the production of the capsular substance. It is also likely that, under certain conditions, some physical property of the medium may render the capsule more readily visible, without causing any real increase of the total amount of capsular material produced (Chapter V).

Because of the highly viscous character which they impart to the media in which they grow, encapsulated bacteria are very troublesome in certain industries, such as sugar refineries. On the other hand, the fact that they often condition the virulence of the pathogenic species renders them of great importance in pathology (Chapter VI). It is not surprising, therefore, that the nature of the capsular material has been the subject of extensive investigation. The capsular substances of a number of bacterial species consist essentially or exclusively of high molecular polysaccharides, often acidic in nature, often possessing acetyl and amino groups, which occur in the natural state as long chain polymers. It is the great asymmetry of their molecule which accounts for the viscosity of their solution and for their anisotropy of flow. These polysaccharides vary in chemical structure, not only from one species to another, but often with each strain within the species. In certain pathogenic groups—the pneumococci, the Friedländer bacilli, the influenza bacilli, for example—the capsular carbohydrates are responsible, because of their difference in chemical structure, for the immunological specificity of the different serological types within each of these bacterial species (Avery, 1932, 1933; Boyd, 1943b; Brown, 1939; Heidelberger, 1927, 1943; Heidelberger, Kendall, and Scherp, 1936; Landsteiner, 1944; Marrack, 1938).

The capsule of the anthrax bacillus and of other aerobic sporu-

lating bacilli seems to be of an entirely different nature, and is made up of a polypeptide of d-glutamic acid (Bovarnick, 1942; Ivánovics, 1938A). It is not known whether the aerobic sporulating bacilli can, under the proper conditions, and like most other bacteria, produce capsular material of polysaccharidic nature, and on the other hand a true polypeptide "capsule" has not yet been found in other microbial species. It may be mentioned in this respect, however, that hemolytic streptococci of group A growing in the "matt" phase produce a serologically specific protein, the M substance, which is so loosely bound with the cell structure that it can be removed from it by trypsin digestion without affecting the viability of the cells so treated (Lancefield, 1943). Whether this M protein can be considered as a capsular material, although it does not manifest itself in the form of a large cellular envelope demonstrable by staining technique, is perhaps only a matter of definition (Chapter IV: 2).

Much has been written concerning the existence of a waxy capsule surrounding the tubercle bacillus. It is true that mycobacteria exhibit hydrophobic and lipophilic properties, and that they contain large amounts of a great variety of lipids, but no evidence is at hand concerning the distribution of these lipids within the cell body. The fact that extraction of tubercle bacilli by lipid solvents fails to alter their staining reactions and morphology until the cellular structure has been altered by drastic treatment, suggests that, if waxes are really a surface constituent of acid fast bacilli, they do not exist as free substances but are combined with other structural components firmly bound to the cell bodies (Fethke, 1938; Knaysi, 1929; Lembke and Ruska, 1940; Wells and Long, 1932. See also Chapter III: 4).

From the cytological point of view, most studies of the bacterial capsule have been concerned with the development of staining reactions. The technical problems involved result from the very poor affinity of the capsular material for ordinary dyes, and from the ease with which it can be washed away during the fixation and washing operations inherent in the staining techniques. Copper salts, methyl or ethyl alcohol, often mixed with acetic acid, are

reagents commonly employed in the capsule staining techniques, and serve to precipitate the capsular substance *in situ*. Direct photography of the pneumococcus capsule has been obtained with the electron microscope, which reveals this structure as a gel of low density surrounding the cell wall (Mudd, Heinmets, and Anderson, 1943).

Only little factual evidence is available concerning the origin of the capsule and its relation to the cellular bodies. Some consider the capsule as a modified outer layer of the cell wall arising from the swelling and gelatinization of its constituents. Others hold that it is a product of secretion, different in nature from the cell wall, and accumulating around it. Medical bacteriologists, impressed by the correlation between virulence and encapsulation, see in the capsule a cellular structure which serves as a protective armor for the pathogen. Still other investigators, recognizing that gums similar in nature to the polysaccharides which constitute the typical capsules are released into the medium by a number of species which do not appear encapsulated, are inclined to believe that capsule formation corresponds in reality to the accumulation around the cell of an excreted product which, on account of its viscosity and of some peculiar properties of the environment, does not diffuse readily into the surrounding medium.

There are a number of observations which are compatible with this last theory. Thus, the fact that capsules usually appear larger and stain better when the bacteria are growing in serum, in tissue fluids, in plant saps, etc., both *in vivo* and *in vitro*, could be accounted for by the greater viscosity of these media and by the presence of proteins which become absorbed or precipitated in the capsular material and serve to increase its stainability. It is known, furthermore, that the mere washing of the microscopic preparation with aqueous solvents during the staining operation is often sufficient to cause disappearance of the capsule, and that the latter can often be removed from the living cells by centrifugation. The capsule in the case of *Erwinia amylovora* is very obvious and sharp in outline if observed in preparations made directly from infected plant exudates negatively stained with India

ink. If, on the other hand, the preparation is mixed with water before drying on the slide, the capsules appear wide, but are less refractive and with the outer edges quite indefinite. Microscopic study of *Erwinia amylovora* reveals not infrequently circular areas of the same shape and dimension as the capsules, but in which the bacterial bodies are located instead of centrally, or from which they are absent. Moreover, although the main part of the capsule does not stain with basic fuchsin, the outer edge often does, probably because of the absorption of some of the constituents of the capsule (Dubos, 1938).

The capsular polysaccharide of type III is readily hydrolyzed by an enzyme which merely depolymerizes it to the aldobionic acid stage (Dubos, 1932, 1935; Dubos and Shaw, 1931). When encapsulated type III pneumococci are treated with this enzyme, the capsule immediately disappears, as evidenced by loss of stainability, without the viability of the bacteria being affected in any way. Furthermore, when the bacteria are transferred to a medium free of the enzyme immediately after treatment, encapsulation showing that the action of the enzyme is to destroy the capsular material but not to affect the basic functions. Similar results have been obtained with other types of attacking the capsular substance of other types of hemolytic streptococci (Dubos and Shaw, 1931; Dubos, 1942; Seastone, 1943; Shaw, 1937; Dubos and Shaw, 1933, 1935, 1941). In fact, the enzyme produced by the bacteria produces an enzyme which renders the capsule readily diffusible by depolymerizing it. This probably accounts for the fact that the capsule is readily visible only in very young cultures. See Chapter VI: 2).

It appears, therefore, that in the case of the bacteria investigated, the bacterial capsule is not destroyed by treatment with neutral aqueous solutions, but is merely depolymerized, resulting in the destruction of the capsular material, without affecting the viability of the bacteria. Although the capsule is not destroyed by treatment with neutral aqueous solutions, it is destroyed by treatment with strong acids, alkalis, or oxidizing agents.

the capsule is not really a cellular structure, but corresponds only to the accumulation around the cell of excreted high molecular material which diffuses very slowly through the medium, some of the antigenic properties of capsulated pneumococci appear to be incompatible with this theory. The capsular polysaccharides of these organisms are not capable by themselves of eliciting the production of antibodies when injected into rabbits, although they react strongly and specifically with the serum of rabbits immunized with the homologous intact bacterial cells. It is known, on the other hand, that type specific, encapsulated pneumococci, killed with heat, formol, or iodine, and washed repeatedly to free them of any capsular material, retain unaltered the ability to induce in rabbits the production of antibodies directed specifically against the free, nonantigenic capsular substance. In other words, the bacterial cells, free of the soluble components of the capsule, still contain some antigenic constituent which has the same specificity as the capsular material (Chapter VII: 4).

It seems probable, therefore, that the cytologist and the immunologist have been discussing, under the name of "capsular material" of pneumococci, two different states of the same substance, one free and excreted outside the cell, where it accumulates in the form of a capsule, the other still bound to the cell, perhaps to the very structure which produces it. We shall consider later the significance of these facts for the understanding of the mechanism of virulence and for the development of immunizing procedures (Chapters VI: 2 and VII: 4). In any event, the bulk of evidence seems to suggest that, in many cases, the structure recognized as a capsule by negative and positive staining procedures consists of excreted material which is no longer a constituent part of the cellular body.

5. FLAGELLA

Although it has been claimed that, in certain bacterial species, the flagella disappear and are replaced by a mucous thickening without loss of motility, and that some of the nonflagellated sulfur

organisms exhibit slow, creeping motion, the motility of bacteria is generally due to the possession of flagella (Gory, 1923; Ullrich, 1926).

These organs vary in size but are always extremely thin, and usually longer than the cells which give them origin. Their diameter falls, in general, below the limits of resolution in visible light, although beautiful cinematography of the motion of the typhoid bacillus and other microorganisms has been achieved by examination in the darkfield, using very bright sunlight as a source of illumination (Pijper, 1938). Many observers feel, however, that observation in the darkfield with ordinary light fails to reveal the presence of flagella in very young cells in spite of evidence of motility, and that these organs become visible only as the cell ages, either because they become thicker, or more likely because they associate in groups (Neumann, 1925; Hofer, 1944; Pijper, 1938). Photographs of the individual flagellar components have been obtained by electronic microscopy, and there is a suggestion that in organisms of the colon typhoid group, the flagella may be tubular in structure. Electronic microscopy of the cells of *Achromobacter harveyi* also reveals two distinct types of flagella of which the larger ones (approximately 40 m μ in diameter) probably consist of several thinner longitudinal units (Hofer, 1944; Johnson, Zworykin, and Warren, 1943; Mudd and Anderson, 1941; Mudd, Polevitsky, and Anderson, 1943; Piekarski and Ruska, 1939).

Their fineness, the low affinity for dyes of their constituent material, and the fact that they are very easily destroyed in ordinary microscopic preparations, greatly complicate the cytological study of these structures. All positive staining methods involve the use of mordants which become deposited on the flagellar surface, thus increasing at the same time the diameter and the affinity for dyes. Although negative staining techniques have also been successful, it is not always easy to establish by any known method that single elements, and not tufts of entangled flagella, are seen in microscopic preparations.

Granted these difficulties of observation, there is general agreement that flagella are helicoidal in shape, forming conic spirals

which exhibit at rest a high curvature of about 50° while they become much straighter and narrower in rapid motion. Their distribution and origin is less certain, and doubt has even been expressed concerning the very existence of peritrichous flagellation which is considered by some workers to result from an artifact produced in the staining of fixed films (Hofer, 1944; Pietschmann, 1939; Pijper, 1938). Whether flagella originate from the membrane, or whether they grow from the cytoplasm, either through openings in the cell wall or from an internal granule comparable to the blepharoblast of flagellated protozoa, is still in dispute. Recent studies of their staining reactions seem to indicate ectoplasmic origin which would explain the persistence of the motility of sporulated bacteria at a time when the spore is already formed and when the cell no longer shows any endoplasmic granulation (Legroux, 1925; Neri, 1940). It is also worth mentioning in this respect that anti-flagellar (anti-H) antibodies precipitate not only on the flagellar surface but also on the cell surface, indicating some similarity in antigenic composition between the two structures (Chapter IV: 2).

Mere mechanical agitation is sufficient to release the flagella from the bacterial cell and complete separation can be achieved by centrifugation or by filtration through candles which retain the cell body but allow passage of the flagella. These simple techniques have been sufficient for the preparation of flagellar material in amounts large enough for immunological studies, but they are not adequate for the preparation of the amounts necessary for chemical analysis.

The flagellar material exhibits an acid agglutination point around pH 4.4, and is probably of protein nature since treatment with heat and with trypsin destroys its ability to react with specific antibody (Beyer and Reagh, 1904; Boivin and Mesrobian, 1938; Malek, 1938; Ogonuki and Abe, 1940; Scholtens, 1938). Some insight into its chemical complexity is given by the phenomenon of phase variation which reveals in the flagellar system of a given bacterial species several antigenic components capable of independent variation. It is not known as yet whether these

different components of the flagellar antigens exist within the same flagellum, or reside separately on the different flagella of the same cell, or whether they result from the fact that a given culture can give rise by variation to individuals differing in flagellar antigenic structure. In any case, it has been found possible through adequate experimental techniques to produce from salmonella strains large numbers of variants characterized by different flagellar antigens which were therefore all potentially present in the parent cultures (Andrewes, 1922; Bruner and Edwards, 1941; Edwards and Bruner, 1939; Gnosspelius, 1939; Kauffmann, 1936c. See Chapter IV: 2).

The motion imparted to bacteria by the flagella has been analyzed by direct microscopic observation. They exert a propeller-like action by a rhythmic contraction moving helicoidally over their surface, thus generating a force which must be considerable, since they can move bacteria at the rate of 200 μ per second (Knaysi, 1938). Movements to right or left are controlled by a change in the angles which the flagella make with the cell body. They act therefore as a rudder as well as a propeller, often following rather than preceding the cell. Direct microscopic examination and cinematography of typhoid bacilli in the darkfield with very bright sunlight illumination reveals that these bacteria swim by means of a long tail. At rest, the tail unwinds itself into two rather broadly coiled spiral flagella which are attached somewhere near the middle of the body of the bacillus and which take up a position at an angle to its long axis. When resuming activity, the two flagella start revolving around their axis, stretch, and become twisted around one another at the rear end of the bacillus where they form the tail (Hutchinson and McCracken, 1943; Pijper, 1938, 1940, 1941b).

At death, each one of the two components of the tail can unwind itself into a large number of very thin threads and this process appears to be irreversible. In fact, these "secondary" flagella dissolve very quickly and can hardly be photographed (Hofer, 1944).

When the flagellated typhoid bacilli are resuspended in a

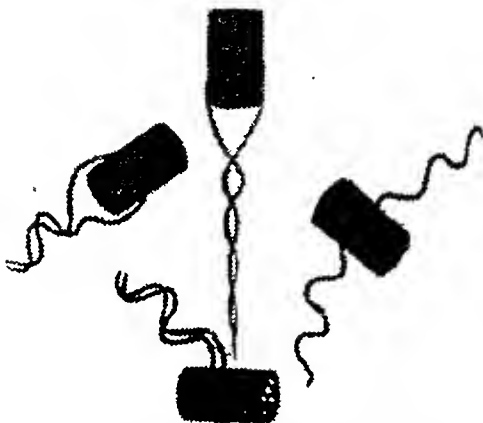


FIG. 5—Diagrammatic representation of motion of typhoid bacilli.
(From Pijper, 1941, fig. 1, p. 398.)

medium containing the flagellar antibody, the flagella become covered with a granular deposit which completely encrusts them. The resulting thickened and stiff spiral structures adhere when

FIG. 6—(See opposite page). a. Typhoid bacilli swimming with long tails, one showing a piece of dirt on its tail. Note dark area between body and tail. b. Typhoid bacilli getting tired. Tails becoming broader spirals. c. Resting typhoid bacillus on the point of unwinding its tail into the two constituent flagella. d. Resting or tired typhoid bacilli. The tail is unwound into the two constituent flagella. This is a reversible change. e. Typhoid bacillus at rest and probably dead, showing its two flagella in a somewhat paralyzed condition. f. The two flagella can unwind into a large number of very thin threads which dissolve very quickly; this process is not reversible. These secondary threads are difficult to photograph and are shown here just before they dissolve. (Microphotographs and legends were obtained through the generosity of Dr. Adrianus Pijper, of Pretoria, South Africa. Methods described in Pijper, 1938, 1940, 1941.)



FIG. 6—Microphotographs of the motile organs of typhoid bacilli taken by special sunlight-darkground technique

fortuitous entanglements occur, and the bacilli become attached to one another, thus causing a loose form of agglutination to take place. This process which has been observed by darkfield microscopy and cinematography has been confirmed by the use of the electron microscope (Mudd and Anderson, 1941; Pijper, 1938, 1941b). As already mentioned, the bacilli agglutinated by anti-flagellar serum show thickened walls and look larger than the normals, and the granular deposits cover the bodies as well as the flagella of the bacillus, suggesting that there exists some constituent which is common to the flagella and to the cellular surface.

Flagella are not essential to the life of the cell. It is possible, for instance, to prevent swarming of *B. proteus* by the addition to the medium of detergents in concentrations which do not affect bacterial growth, but which either destroy the flagella or prevent their formation (Lominski and Lendrum, 1942). Furthermore, the flagellated strains of all bacteria give rise to nonflagellated variants and this loss is often of a permanent character similar to a mutation (Neri, 1940). We have already mentioned another type of variation resulting not in the loss of the flagella but in a change in their chemical composition, which is reflected in the reversible change in antigenic structure known as phase variation (Chapters IV: 2 and V: 4). These modifications of a transient or permanent nature, as well as the occurrence of typical flagella in representatives of bacterial groups (cocci) not usually regarded as possessing motility, indicate that, as in the case of endospores, much remains to be learned of the occurrence and importance of flagellation in the bacterial world (Gibson, 1935; Jordan, Caldwell, and Reiter, 1934; Kobsmüller, 1935).

6. CELLULAR DIVISION AND COLONIAL MORPHOLOGY

Several of the bacterial structures which have been considered in the preceding pages, such as spores, capsules, flagella, etc., are not essential or permanent attributes of the normal individuals of a given species, but occur only in certain cultural phases or under certain conditions of cultivation. In fact, all bacterial cultures,

even issued from single cells, can give rise to a number of variant forms which become stabilized through a number of successive generations and which exhibit extreme variations in cellular morphology (Chapter V: 4, 5, 6). These cellular differences are so often correlated with differences in the appearance of colonial growth on solid media, that the same terminology can be used to a large extent for the simultaneous description of cellular and colonial variation.

The concept of "bacterial dissociation" developed from the observation that most bacterial cultures can produce several types of colonies consisting of organisms different in physical, chemical and biological properties. It may be useful to summarize at this time in very general terms, and as far as it is possible to generalize, the different morphological characters associated with each of the different dissociative phases exhibited by bacteria, and to consider later how the properties of the individual cell affect the growth and the morphological organization of the colony (Hadley, 1927, 1937, 1939A).

The mucoid (M) phase of a bacterial culture is characterized by the viscous consistency of the colony which can, however, appear watery or even transparent; these differences are probably related to the amount and nature of the capsular material produced by the culture. The cells are usually very short, almost coccus-like, even in the bacillary species.

The smooth (S) colonies are round, convex, opaque, and possess an even margin and a smooth to glistening surface. In bacillary species, the cells are often longer than in the mucoid phase.

The rough (R) colonies are larger, flat, irregular in shape, with a rhizoid or filamentous border. The surface always possesses some degree of roughness. The cells are long and filamentous, often of a twisted mycelial appearance.

Many species also produce the small G colonies, which are often unstable and consist of minute individual cells or structures which have been regarded as gonidia by some investigators (Duff, 1937, 1939; Flynn and Rettger, 1934; Haag, 1927; Haddow, 1938; Hadley, 1927, 1937, 1939).

Finally, in some species, especially in certain streptococci and in the tubercle bacilli, there has been described a D phase consisting of diphtheroid forms (Mellon, 1920, 1921; Morton, 1940; Morton and Sommer, 1944).

It has been found possible in a few cases to relate the organization of the colony to the mode of growth of the constituent cells. The final separation of two bacteria following cell division can be achieved by post fission movements of three different types, the so called snapping, slipping, or whipping movements. These three different types of movements used to be considered as characteristic of three different bacterial groups, and to occur respectively in the aerobic spore formers, the coliform bacilli, and the diphtheroids. More recent studies seem to indicate, on the contrary, that each type of movement can occur in any species and is characteristic not of the bacterial group, but of the dissociation phase—muroid, smooth, rough—in which the organisms happen to be.

In the snapping type of post fission movement, the two individual cells push against each other, bending in the middle. The plasmodesmids (the protoplasmic bridges which unite the two dividing cells) become tense and finally snap, thus completing cellular division. Separation of cells by snapping is characteristic of organisms growing in chains, and may occur in any part of the chain, causing it to break. The two new chains then continue to grow independently, and give rise to the loops and festoons seen at the edge of colonies of the type produced by *Bacillus anthracis*. This type of post-divisional fission was first recognized with the anthrax bacillus and with other aerobic spore formers, and was associated with the medusa-bead type of colony which is composed of threads and chains. In reality, however, the same sequence of post fission movements, resulting in the same colonial structure, can also be seen in the coliform bacilli when they are observed in the rough phase. The cells which constitute the rough colonies in this group of organisms appear as long rods, with square-cut ends, and strong polar attachments to one another; the rough colony, instead of consisting of small, separate organisms, is made up

of bacilli lying together in threads almost forming a mycelial structure (Bisset, 1938; Roelcke and Bartram, 1939; Roelcke and Intelkofer, 1938; Stapp and Zycha, 1931).

When, on the contrary, coliform bacilli are observed in the smooth phase, they appear as short rods with rounded ends and little attachment to their neighbors. Following cell division, they immediately separate and one of the two glides partially past the other (slipping movement), with the result that they show no characteristic arrangement within the colony.

Post fission movements are still of a different character in the corynebacteria; one cell moves through an arc of a circle, the center of which is the point of attachment of the two cells, the moving cell forming the radius and never bending. The movement may be rather sudden and is designated as whipping motion; it tends to bring the cells in rows and bundles parallel to one another, giving rise to the so called palisade arrangement.

In addition to the strength of longitudinal attachment between the individual bacteria, and the consequent rigidity of the chains or filaments which they may form, there are other cellular properties which affect colonial appearance. The nature and abundance of the capsular material obviously gives to the mucoid colony its typical character; the deficiency in metabolic activity of its component cells probably determines the small colonial dimensions of the G variant (Flynn and Rettger, 1934). In cultures of *B. mycoides*, some unidentified intimate property of the protoplasm causes the threads to turn either clockwise or counter clockwise, according to the strain, thus giving rise to dextral or sinistral colonies (Gause, 1939; Stapp and Zycha, 1931).

It must be remembered, on the other hand, that many of the differences which we have considered are not of the all or none type. There seems to exist between the pure R and the pure S variants a variety of forms which are intermediate not only in colonial morphology, but in other biochemical and biological properties (Paul, 1934). Finally, it is obvious that environmental factors can also affect the appearance of the colony. Thus, the

amount of free moisture conditions the mucoid character of growth on agar, and the mechanical resistance offered by the medium ultimately affects the form of the colony by modifying the course of the growing bacillary threads.

In spite of the difficulties encountered in any attempt to correlate cellular and colonial morphology, some authors feel that the time has come to unify and standardize the terminology employed for the description of the phenomena of dissociation in the different bacterial groups. It has been pointed out, in particular, that the symbols employed to designate the different dissociative phases of pneumococci are not consistent with those employed in the description of other species. According to this view, encapsulated pneumococci should be described as being in the M (mucoid) and not in the S (smooth) phase, as has been the usual practice, and the pneumococci formerly called R (rough) should really be considered as S (smooth), since they do not correspond in morphology to the R forms of other bacterial types (Dawson, 1934). In fact, it is possible to isolate from old pneumococcus cultures a variant form, long unrecognized, which gives on agar a wrinkled colony and filamentous mycelium-like growth typical of the R forms of other species (Shinn, 1937). The terminology employed for describing streptococci could also be modified according to the same principles (Dawson, Hobby, and Olmstead, 1938).

There is as yet no convincing information concerning the true nature and significance of bacterial dissociation, and it is not known whether the M, S, R, G, D variants correspond to stabilized phases in some orderly developmental life cycle of a given strain, or whether they are only examples of discontinuous variations, mutation-like in nature (Chapter V: 4-6). Even the mere description of these variant stages is far from complete, and the occurrence of so many intermediary forms renders it more difficult and more confusing. It is not possible, therefore, to adopt at the present time a final terminology which would be adequate for all the phenomena under consideration. On the other hand, the striking similarity in the dissociative patterns exhibited by all

bacterial species (as well as by yeasts and perhaps by algae), suggests an essential similarity of the mechanism of variation in all these microorganisms, and much would be gained by describing the observational data with a more uniform terminology than has been used heretofore (Hadley, 1927, 1937, 1939A).

III

PHYSICOCHEMICAL AND STAINING PROPERTIES OF BACTERIA

*I'll tell thee everything I can:
There's little to relate,
LEWIS CARROLL*

1. PHYSICOCHEMICAL BEHAVIOR OF BACTERIA

THE minute size of bacteria has permitted the utilization of some of the methods of colloid chemistry for the study of their behavior. Bacteria in suspension exhibit brownian movement; their migration in an electrical field proceeds according to a characteristic pattern conditioned by the ionic environment and the chemical structure of the cells under investigation; the stability of their suspensions in aqueous media also depends on the ionic environment and on the presence of protective colloids. It is obvious that the laws which govern the behavior of inorganic colloids and of proteins are not entirely applicable to structures as highly organized and as chemically complex as living cells. The different cell membranes insulate the cytoplasm against certain modifications of the environment, and the ionic phenomena in the living protoplasm are not only exchange reactions, but are also the expression of complex metabolic events. Thus the accumulation of potassium within the cell and its release into the surrounding medium are correlated respectively with the synthesis and breakdown of polysaccharides (Pulver and Verzár, 1940). Phosphoric acid undergoes a cycle of changes from the inorganic to the organic form during carbohydrate metabolism, and energy is stored by the cell in the form of phosphoric acid esters (Lipmann, 1941). Death results in a profound modification of permeability properties, and

particularly in the loss of ability for the selective accumulation of both organic and inorganic materials. Even after the cytoplasmic membrane has been destroyed, however, there remain other membranes or cell walls which modify the rate of passage of certain molecules from and to the environment (Frey-Wyssling, 1938, p. 154).

Although it is impossible to separate the colloid behavior of bacteria from their metabolic activities, an attempt will be made in the following pages to recognize a few physicochemical reactions which may be of some help in defining the relation of the cell to the environment.

Effect of Ions and Colloids on the Stability of Bacterial Suspensions.—The ability to form stable suspensions or to agglutinate spontaneously in aqueous saline solutions has been used as one of the descriptive characters of bacterial cultures. This property, however, is not a constant character of the species, but on the contrary varies profoundly according to the dissociation phase under which the culture happens to be (Arkwright, 1920). In general, cultures in the mucoid or smooth phase form more stable suspensions than do the rough variants which are apt to auto-agglutinate especially in the presence of electrolytes. This difference in behavior has been attributed to the presence in the mucoid and smooth forms of complex polysaccharides which, because of their hydrophilic properties and perhaps also on account of the strong negative charge which they impart to the cell, increase the stability of the suspension (Chapter IV: 2). There are, however, many other aspects of cellular structure which can affect the stability of cell suspensions. The morphology of the cell is probably a significant factor in this respect, since rough variants often grow in long filamentous forms which become at times almost mycelial in character, whereas the mucoid or smooth cultures consist of shorter individuals which remain separated from each other (Chapter II: 6).

In a number of cases the auto-agglutinability of bacteria is due to the presence of a substance which confers hydrophobic properties upon the cell. For instance, the R forms of a number of

Gram-negative microorganisms (*vibrios*, *shigella*, *salmonella*) which exhibit spontaneous agglutination in the presence of electrolytes, give stable suspensions after the cells have been extracted with alcohol or chloroform. The extraction process removes a lipid (probably a phospholipid), soluble in these solvents and insoluble in aqueous saline solutions, and which is probably responsible for the spontaneous agglutinability since the extracted stable bacterial suspension becomes unstable again in the presence of electrolytes when this substance is again added to it. It appears, therefore, that the lipid is normally present on the surface of the rough cells of several species of Gram-negative organisms and confers upon them its hydrophobic character. A similar (if not identical) substance also exists in the smooth forms; in this case, however, the polysaccharide of the specific O antigen probably neutralizes the effect of the phospholipid, perhaps because it occupies a more superficial position, or by virtue of its hydrophilic property and of its strongly negative charge (White, 1927, 1928).

There also exists in *salmonella* a protein, the Q substance, soluble in acid alcohol, but insoluble at neutrality in physiological salt solution, which may play some part in determining the instability of the suspensions of cultures in the R phase (White, 1932). The matt forms of hemolytic streptococci of group A exhibit also frequently spontaneous agglutination; the substance responsible for lack of stability in these organisms can be removed by proteolytic digestion (which destroys the M protein) without affecting the viability of the cell (Lancefield, 1943. See Chapter IV: 2).

The stability of bacterial suspensions is affected not only by the protective colloids which are the products of the cell, but also by substances which it adsorbs from the medium. Thus, at pH levels between 3 and 3.5, the negative charge of *E. coli* is so reduced by low concentrations of positively charged gelatin that the cells agglutinate. As the concentration of gelatin increases, the larger amounts adsorbed give the bacterium an increased positive charge and render the suspension stable again. At still

higher pH levels (4 to 4.5), the negative charge of the cell is higher and the positive charge of gelatin lower; larger amounts of gelatin are then required to render the bacterial suspension unstable. In the highest concentrations, the gelatin forms a complete layer on the surface of the bacterium and the stability of the suspension is therefore independent of the surface potential. Gelatin and edestin affect the stability of suspensions of *E. coli* in such great dilution that it is apparently sufficient to cover, not the whole cell, but only localized patches of it, to affect its stability (Eggerth and Bellows, 1922).

Since the ability of ions to agglutinate bacterial systems is related to their place in the Hofmeister series, and since ions can quantitatively displace each other on the bacterial cell by exchange reactions, it is obvious that the ionic composition of the medium is also important in conditioning the stability of bacterial suspensions.

Acid Agglutination of Bacteria.—The fact that certain bacterial cultures agglutinate in fairly well defined pH zones has suggested the use of the "acid agglutination point" as one of the descriptive characteristics of bacterial species. As in the case of agglutination by electrolytes, however, this value is not constant for the species but varies from one cultural phase to the other. In general, non-specific variants in the R phase exhibit a zone of least stability around pH 4.0 to 4.5. This property is due in part to the fact that the nucleic acids of the cell form insoluble salts with many proteins and with other compounds in this very pH range. The specific antigens often protect the smooth and mucoid forms against agglutination at acid reactions.

Acid agglutinability has also been traced to cellular components other than nucleoproteins. Thus, the typhoid bacillus presents two fairly well defined acid agglutination zones, one at pH 4.4 to 4.6, the other at pH 2.3 to 2.9. The former seems to be associated with the presence of the H antigen (the constituent of the flagella), and the flagellar material separated from the cell does, in fact, precipitate in this pH zone. The second agglutination point is correlated with the presence of the Vi antigen, although direct proof

is not yet available that this cellular component is truly insoluble at pH 2.3 to 2.9 (Malek, 1938; Ogonuki and Abe, 1940; Scholtens, 1938) (Chapters II: 5 and IV: 2). Tubercle bacilli, either intact or ground up, are most readily agglutinated at pH 2.8 to 3.0; extraction by lipid solvents of the ground-up bacilli leaves a cellular residue which precipitates at pH 2.8, whereas the extracted lipid material can be precipitated at pH 1.0 to 1.5. These results suggest that the surface of the tubercle bacillus possesses an exposed protein component and does not necessarily consist of a waxy capsule (Freund, 1925) (Chapter II: 4).

Electrokinetic Mobility of Bacteria.—Attempts have also been made to use the electrokinetic mobility of bacteria as a descriptive character, and even as an index of the comparative virulence of the different members of one species. It must be emphasized, however, that in addition to the experimental difficulties attending the determination of this property, there are many variables which modify the migration of bacteria in an electric field. Thus, it is generally agreed that young cells are much more electronegative than adult cells, and it is even probable that the modifications of electrical charge during the growth cycle are extremely complex (Choucroun, 1938; Moyer, 1936; Winslow, Falk, and Caulfield, 1923). On the other hand, each one of the surface cellular constituents contributes to the electrokinetic mobility, the rate and direction of which consequently vary as the culture undergoes variation. For instance, both the Vi and O antigens of *Eberthella typhosa* carry a negative charge over a wide range of pH, and the rate of migration is determined by the Vi component when both antigens are present on the same cell (Combiesco and Soru, 1939). In general, the specific antigens—often of a polysaccharide acid nature—which characterize the smooth and mucoid variants, determine the behavior of the latter in the electric fields. It is likely indeed that the claims that virulence is correlated with extreme electronegativity rest upon the fact that the latter property is an expression of the S or M dissociative phases of the bacterial cultures (Stearns and Roepke, 1941).

At physiological pH, bacteria normally carry a negative charge

which can be neutralized by the addition to the system of sufficient hydrogen ions (Kendall, 1925; Tittsler and Berry, 1938). In the case of *B. cereus*, change in the direction of cataphoretic migration occurs at pH 3.0 and it is of interest that this isopotential point coincides with the "iso-electric" point determined by the ability

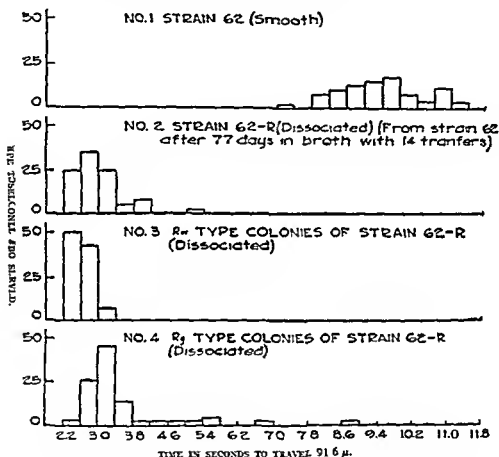


FIG. 7.—Histograms of velocities of *Brucella abortus*. (From Stearns and Roepke, 1941, fig. 2, p. 750.)

of the same organism to retain acid and basic dyes at different reactions (Stearn and Stearn, 1925; Winslow, Falk, and Caulfield, 1923). It is possible that careful study of the electrokinetic behavior of bacteria under different experimental conditions would supply information concerning some of the properties of their constituents, and the fact that the migration velocity remains unaltered after the death of the cell renders the technique more

likely to be amenable to experimental study (Winslow, Falk, and Caulfield, 1923). The possibilities of the method are illustrated by an analysis of the effect of p-aminobenzoic acid and of sulfonamides on the electrokinetic mobility of *E. coli*, which indicates that the drugs associate with the organisms through their aromatic amino groups (Chapter VIII:1), and by a comparative study of several staphylococcus strains, some of which do, while others do not, exhibit properties indicating amphoteric surfaces (Bradbury and Jordan, 1942; Verwey and Frobisher, 1940).

Uptake of Ions by Bacteria.—The direction of migration of bacteria in an electric field, the spontaneous agglutination which they often exhibit at acid reactions, their great affinity for basic dyes, indicate that these organisms usually exhibit a preponderance of acidic over basic groups under physiological conditions of pH.

Because of their negative charge and of their colloidal dimension, their relationship to the positively charged ions in the environment presents a special interest. It is readily observed indeed that ionic exchanges take place between the cell and the environment, and that the interchange is conditioned by the concentration of the ions as well as by their degree of adsorbability. This phenomenon of cation exchange is illustrated in the following experiment, in which the amount of adsorbed magnesium replaced from bacterial cells by a number of other cations was demonstrated by direct titration.

Magnesium sulfate was added to a suspension of *E. coli* to equal approximately 0.001 M solution. After allowing sufficient time for the magnesium to become adsorbed, the cells were washed until free of dissolved magnesium. To 25 ml. portions of the bacterial suspensions were added, respectively, 1 ml. of 0.1 N NaCl, KCl, CaCl₂, BaCl₂, MnSO₄, HCl. These suspensions were shaken thoroughly and then centrifuged. The control showed a very slight amount of replaced magnesium. Ions like Na and K, which are adsorbed slightly or not at all, replaced such a small amount of magnesium that it was not measurable by this method. Other ions which are more strongly adsorbed replaced the magnesium. The

values obtained by replacing the adsorbed magnesium compare favorably with those obtained by measuring the adsorbed hydrogen. These values are shown in Table 1 (McCalla, 1940).

TABLE 1

REPLACEMENT OF ADSORBED MAGNESIUM BY OTHER CATIONS

AMOUNT OF ADSORBED MG REPLACED BY VARIOUS CATIONS (M.eq./100 gm. Bacteria)						
Control	Na	K	Ca	Ba	H	Mn
Very slight	Slight	Slight		16.5	26	
Very slight	Slight	Slight	38	38	50	38
Very slight	Slight	Slight	54	54		54

Data from McCalla (1940, Table 4, p. 29).

In order to demonstrate that bacterial systems are capable of exchanging other ions for hydrogen, cells of *Bacillus bellus* and *Escherichia coli* were washed and enough dilute HCl added to adjust the bacterial suspensions to pH 4.0 and 5.0 respectively. These "H-bacteria" were then washed with distilled water and 1.0 ml. of 0.1 N solution of various electrolytes added to 5.0 ml. of the bacterial suspensions. Data presented in Table 2 show that whereas ions like Na⁺ and K⁺ replace little hydrogen, other ions like Ag⁺ and Hg⁺⁺ are very active from this point of view. *E. coli* adsorbed 22.3 m.eq. of H⁺ per 100 gm. of bacteria; Na⁺ displaced 1%, Ca⁺⁺ 3%, and Ag⁺ 15% of the adsorbed H⁺; the activity of the cation is not influenced appreciably by the nature of the anion present in the system (McCalla, 1941b).

Although the examples of exchange reactions considered so far concern inorganic ions, similar observations can be made with organic substances. Thus methylene blue, a basic dye, should behave as a cation and compete with other cations, Mg, for instance, adsorbed on the bacterial cell, according to the following equation:

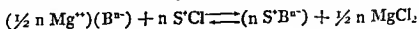


TABLE 2
 THE DISPLACEMENT OF H^+ FROM *H*-BACTERIA BY OTHER IONS*

ELECTROLYTE		<i>E. coli</i>					<i>B. bellus</i>		
		pH of Solution	pH of Electrolyte + <i>H</i> -bacteria	Decrease in pH due to <i>H</i> -bacteria	H^+ replaced $\times 10^{-4}$	M. eq. H^+ replaced/100 gm. bacteria	pH of Electrolyte + <i>H</i> -bacteria	Decrease in pH due to <i>H</i> -bacteria	H^+ replaced $\times 10^{-4}$
<i>H</i> bacteria									
NaCl	5.00	5.00	5.00	—	—	22.3	4.12	—	—
KCl	5.20	4.56	4.56	0.44	0.17	0.21	4.18	0.02	0.05
CaCl ₂	5.43	4.56	4.56	0.44	0.17	0.21	4.12	0.08	0.16
BaCl ₂	5.00	4.17	4.17	0.83	0.56	0.71	3.75	0.45	1.17
HgCl ₂	5.10	4.22	4.22	0.78	0.49	0.62	3.78	0.42	1.03
Al ₂ (SO ₄) ₃	4.63	3.68	3.68	1.32	1.90	2.43	3.39	0.81	3.40
LiNO ₃	4.05	3.71	3.71	1.29	1.80	2.32	3.49	0.71	2.69
KNO ₃	5.70	4.56	4.56	0.44	0.17	0.21	4.06	0.04	0.08
Ca(NO ₃) ₂	5.60	4.63	4.63	0.37	0.13	0.16	4.06	0.04	0.08
Ba(NO ₃) ₂	5.30	4.27	4.27	0.73	0.42	0.53	3.88	0.22	0.51
Pb(NO ₃) ₂	5.22	4.31	4.31	0.69	0.38	0.48	3.90	0.20	0.42
AgNO ₃	4.10	3.61	3.61	1.39	2.30	2.94	3.45	0.65	2.78
	4.20	3.51	3.51	1.49	2.90	3.71	3.22	0.88	5.36

Data from McCalla (1941b, Tables 3 and 4, pp. 780-781)
 * For experimental details, see text, page 60

(where B stands for bacterial material, and S for the stain). It should be possible, therefore, to measure the displaced Mg chemically and to show a stoichiometrical relationship between the methylene blue adsorbed and the Mg displaced.

Bacteria containing large amounts of adsorbed Mg were obtained by growing *E. coli* in the presence of 0.5 per cent $MgSO_4$, or by adding $MgSO_4$ to a suspension of *Staph. aureus*. The cells were then washed free of unadsorbed Mg and aliquots of the washed suspension treated with known quantities of 0.01 M methylene blue. Determination of the Mg released in solution from the bacteria revealed that methylene blue is capable of replacing the inorganic ion, as indicated in Table 3 (McCalla, 1941a).

TABLE 3

THE DISPLACEMENT OF MAGNESIUM FROM *E. coli* AND *St. aureus* WITH METHYLENE BLUE

TEST ORGANISMS	AMT. OF Mg IN METH. BLUE	M.EQ.* Mg REPLACED/100 GM. BACTERIA†		M.EQ.* OF METH. BLUE ADSORBED PER 100 GM. BACTERIA
		Water + Bact.	Meth. Blue + Bact.	
<i>E. coli</i>	00	trace	21.4	22.8
<i>E. coli</i>	00	trace	38.5	60.8
<i>St. aureus</i>	00	trace	17.3	30.5
<i>St. aureus</i>	00	trace	10.9	24.1

Data from McCalla (1941a, Table 1, p. 29)

* Refers to milligram equivalents.

† Expressed as weight of cells dried at 105° C.

If the experimental system is so arranged that the ions displaced are predominantly adsorbed hydrogen, the exchange reaction results in an increase in H^+ concentration which can be readily measured in terms of pH (McCalla, 1941a) (Table 4).

The exchange reactions which we have considered do not apply only to cations, but also to anions. Just as basic dyes by displacing hydrogen from the adsorption sphere of the bacterial cell increase

TABLE 4

THE DISPLACEMENT OF HYDROGEN WITH BASIC FUCHSIN FROM *Staphylococcus aureus* AND *Escherichia coli*

ML OF H-BACTERIA	ML. OF STAIN	ML. OF WATER	PH OF H-BACTERIAL SUSPENSION			
			<i>Staph. aureus</i>		<i>Esch. coli</i>	
			Actual pH	Decrease in pH	Actual pH	Decrease in pH
100	0.00	5.00	6.00	0.00	4.50	0.00
00	5.00	10.00	6.10	0.00	6.20	0.00
100	0.10	4.90	5.80	0.20	4.55	+ 0.05
100	0.25	4.75	5.70	0.30	4.38	0.12
100	0.50	4.50	5.50	0.50	4.25	0.25
100	1.00	4.00	5.25	0.75	4.00	0.50
100	2.00	3.00	5.00	1.00	3.80	0.70
100	3.00	2.00	4.65	1.35	3.75	0.75
100	4.00	1.00	4.25	1.75	3.72	0.78
100	5.00	0.00	4.15	1.85	3.72	0.78

Data from McCalla (1941a, Table 5, p. 31)

the H^+ concentrations of the suspension, so acid dyes have the opposite effect because of their ability to displace anions (McCalla, 1941a) (Table 5).

TABLE 5

THE INFLUENCE OF AN ACID DYE UPON THE PH OF *Escherichia coli*

ML OF H BACTERIA	ML. OF STAIN	ML. OF WATER	pH OF H-BACTERIAL SUSPENSION TREATED WITH PHLOXINE	
			Actual pH	Increase in pH
50	0.00	2.00	4.90	0.00
50	0.10	1.90	6.08	0.78
50	0.25	1.75	6.20	0.90
50	0.50	1.50	6.23	0.93
50	1.00	1.00	6.15	0.85
50	2.00	0.00	6.00	0.70
00	5.00	2.00	5.30	0.00

Data from McCalla (1941a, Table 6, p. 31).

The foregoing phenomena establish that the bacterial cell is the site of very active ionic exchange reactions which approach stoichiometrical relationship, and that organic ions (at least as illustrated by the case of dyes) compete with inorganic ions for the same positions in the cell. These facts are of utmost importance in defining the reactions between cell and environment. It is often observed, for instance, that bacteria tend to adjust the pH of the medium by mechanisms which appear other than the production of acidic or basic metabolites; thus, the pH increases from 3.4 to 6.1 when a suspension of *E. coli* is added to an acid solution, a result which can probably be explained in terms of the exchange reactions just described (Winslow and Falk, 1923). The adsorbability of an ion is reflected in its toxicity; ions such as Na^+ and K^+ which are only weakly adsorbed are not toxic in dilute solutions, whereas H^+ , Ag^+ , Hg^{++} , etc., are more strongly adsorbed and much more toxic. Further consideration will be given later to this problem when it is pointed out that mere displacement of toxic by other less toxic ions is often sufficient to restore the viability of a cell apparently "killed" by certain antiseptics (Chapter VIII:1 and 2). It will be shown presently, on the other hand, that the activity of dyes as cations or anions conditions their behavior in the staining reactions.

2. MECHANISM OF STAINING

Physical and Chemical Theories of the Phenomena of Staining.
—The retention of dyes by fabrics has been ascribed to reactions due to solution, to adsorption, or to chemical forces. The "solution theory" assumes that the dye molecule passes into the dyed substance, distributing itself as between two immiscible solvents in each of which it is soluble. This theory never found many followers among cytologists, probably because the cell presents too great a structural complexity and too many intracellular phases to permit such a simple statement of the problem. According to the "adsorption theory," staining results from the deposition and the retention of the dye on surfaces by forces chiefly physical in

nature. Proponents of the "chemical theory," on the other hand, postulate that there occurs between the dye and the stained substance a definite stoichiometric reaction which gives rise to the formation of reaction products of definite molecular properties; in general, the color ion of the dye is supposed to form a salt with some reactive group of the stained substance (Holmes, 1929; Stearn and Stearn, 1929a, 1930b).

It is likely that, in the case of biological material at least, the great variation in reactivity of the substances to be stained and the unknown behavior of the dye molecule at the surfaces of complex organic molecules render difficult any general statement of the phenomena involved. The importance of cellular structure in the mechanism of staining is illustrated by the behavior of Congo red. This dye penetrates the membrane of young living bacterial cells, but does not stain the protoplasm. With increasing age of the culture, some of the cells become uniformly stained, whereas cultures killed by formol or other fixatives do not become stainable. Cultures killed at 60° C. stain more deeply and uniformly than those killed at boiling temperatures. It would appear, therefore, that mere death of the cells is not the fact which causes them to become stainable, and that the change of behavior toward Congo red is due to some process which occurs as a result of partial autolysis (Henrici, 1928, p. 55).

Generalizations concerning the mechanism of staining become even more dangerous when the staining technique involves several independent steps and in particular the use of mordants. On the other hand, the longstanding controversy between the proponents of the physical and chemical theories has lost much of its meaning, since it is now realized that the phenomena of colloid chemistry are governed by the same forces which operate in solution chemistry, only modified in so far as they occur on surfaces. Instead of attempting to differentiate between chemical and adsorption mechanisms of staining, therefore, it appears more profitable to describe some of the specific factors which have been recognized to affect the outcome of the staining process.

Staining Considered as a Result of Ionic Exchange.—The im-

portance of acidity or alkalinity has been empirically recognized in practically all staining techniques since there are few of the formulae which do not recommend the addition of inorganic or organic acids or bases to the staining reagents. In general, staining with acid dyes is more effective at acid reactions, whereas staining

cc% HCL	40.0	20.0	10.0	5.0	2.0	1.0	0.5	0.2	0.1	0
PH	0.55	0.75	1.2	1.3	1.8	2.1	2.4	2.7	3.0	4.3
TYPHOID										
B. Coli ²										
B. Coli ³										
B. Coli ⁴										
B. Coli ⁵										
B. Coli ⁶										
STAPH.										

— STAINED BY METHYLENE BLUE

— STAINED BY FUCHSIN S (ACID)

— 50% STAINED BY METHYLENE BLUE ONLY

FIG. 8.—Results of staining different bacteria with the methylene-blue-acid-fuchsin mixture at different pH values. (From Tolstoubov, 1929, chart 3, p. 85.)

with basic dyes is more intense in the alkaline range, as appears from the following observations.

When bacteria are treated at different pH with mixtures of basic and acid dyes, methylene blue and eosin or acid fuchsin for example, they take the acid dyes only at extreme acid reactions, whereas they all retain methylene blue when the medium is neutral or alkaline. At intermediate pH, different bacteria exhibit different affinities for the two types of dyes, and this property has

been used for an approximate measurement of the iso-electric point of the protoplasm of each strain (Tolstouhov, 1929).

A more quantitative expression of the same phenomenon can be obtained by measuring the amount of acid and basic dye retained by bacterial suspension at different pH. When crystal violet or acid fuchsin (0.01 M solutions) is added to washed suspensions of *Corynebacterium simplex* and *Bacillus bellus* at reactions ranging from pH 0.60 to pH 9.10, determination of the amount of dye remaining in solution after removal of the cells by centrifugation gives a measure of the amount of dye taken up as a function of pH. The results presented in Table 6 show that the amount of crystal violet taken up increases enormously as the reaction becomes more alkaline, whereas the opposite is true with acid fuchsin (McCalla and Clark, 1941).

TABLE 6

THE ADSORPTION OF CRYSTAL VIOLET, ACID FUCHSIN, AND H^+ AT DIFFERENT pH VALUES BY *Corynebacterium simplex* AND *Bacillus bellus*
(as M Mol. per 100 G Bacteria)

CRYSTAL VIOLET				ACID FUCHSIN				H^+			
<i>C. simplex</i>		<i>B. bellus</i>		<i>C. simplex</i>		<i>B. bellus</i>		<i>C. simplex</i>		<i>B. bellus</i>	
pH of dye-bacteria	m. mol. adsorption	pH of dye-bacteria	m. mol. adsorption	pH of dye-bacteria	m. mol. adsorption	pH of dye-bacteria	m. mol. adsorption	pH of HCl-bacteria	m. mol. adsorption	pH of HCl-bacteria	m. mol. adsorption
0.58	19.0	0.80	0.0	0.52	15.6	0.89	21.5	0.75	119.0	1.36	77.2
1.09	30.8	1.30	3.1	1.00	20.2	1.49	30.4	1.35	116.0	2.65	75.0
1.62	51.3	1.82	14.1	1.50	21.0	1.90	31.2	3.80	64.5	4.20	48.6
1.96	68.6	2.17	24.6	2.00	17.6	2.30	33.3	6.00	22.0	5.35	25.2
2.46	80.0	2.70	20.8	2.54	11.6	2.85	30.5	6.80	16.1	6.00	20.0
2.84	86.6	3.35	31.8	3.15	10.2	4.00	14.2	—	—	6.55	15.5
3.65	95.0	4.20	63.3	4.40	7.4	4.95	7.4	—	—	—	—
3.91	99.6	5.12	98.3	4.91	5.0	5.10	5.1	—	—	—	—
5.95	109.0	6.30	106.0	6.73	4.6	7.50	1.4	—	—	—	—
8.40	109.0	7.79	109.0	—	—	—	—	—	—	—	—

Data from McCalla and Clark (1941, Table 1, p. 97).

These findings can be considered as further examples of the ionic exchanges discussed in preceding pages; they illustrate again that adsorption of crystal violet displaces adsorbed H^+ , since the mixtures of dye solution and bacteria exhibit pH values from 1 to 2 pH units lower than the values of either the dye or the bacterial components of the system taken separately (McCalla and Clark, 1941).

Cellular Factors Affecting Staining with Acid and Basic Dyes.—The reactions which occur between the dye and the cell can be explained on the assumption that the latter consists of an equilibrated system of two classes of ampholytes, or, for the sake of simplicity of analysis, of two ampholytes, one of which is distinctly more acidic than the other. When such a system is at a pH more acidic than that corresponding to the iso-electric point of the more acid component, both act as acids and combine with cations. On the other hand, one component acts as a base and the other as an acid when the H^+ concentration lies between the two iso-electric points. In the case of simple systems, it is possible to calculate an intermediate "iso-electric point" from the acidic and basic strengths of the two components and from their relative amounts. Although such calculation is impossible in the case of systems as complex as living cells, indirect methods, such as titration curves of bacterial suspensions, direction of migration of the cells in an electric field at different pH, relative affinity for anions and cations, permit the determination of an approximate value which has been termed the "iso-electric zone" of the cell (Stearn, 1933; Stearn and Stearn, 1928a, 1930b, 1931a, b).

This iso-electric zone has been recognized chiefly by determining the pH range at which the cell changes its affinity from the basic to the acid dye. Examples of results obtained by staining with mixtures of dyes have been illustrated in Figure 8 (Tolstouhov, 1929). Similar values can be obtained by staining bacteria with pure solutions (not mixtures) of acid or of basic dyes, washing the stained preparations with buffers of known pH and finally decolorizing with acetone. When *Staphylococcus aureus*, *Corynebacterium diphtheriae*, and *Escherichia coli* were studied by this

method, using gentian violet and carbol acid fuchsin as basic and acid dye, reversal of the staining reaction occurred respectively at pH 2-3, 4-5, and 5-6, for these three bacterial species (fig. 9) (Stearn and Stearn, 1924a).

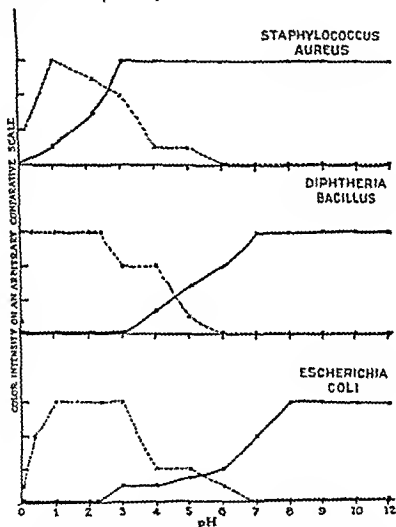


FIG. 9.—Showing the retention of acid (broken line) and basic (unbroken line) dye at varying values of pH (From Stearn and Stearn, 1924, fig 1, p 471.)

It is clear that the so-called "iso-electric" values defined above do not refer to a point at which neither cation nor anion is retained, but only to the zone in which there appears to be fairly equal

retention of anion and cation. These iso-electric points vary not only from culture to culture, but also to some extent from cell to cell in the culture, and even from one structural component to another within a given cell. Young bacteria, like embryonic tissues, exhibit a greater affinity for the basic dyes than do older cells, probably because of their higher content of nucleic acid (Caspersson and Schultz, 1939; Frey-Wyssling, 1938, p. 174; Henrici, 1928, p. 97; Tolstouhrov, 1929).

In any case, the preponderance of acidic over basic groups in all bacterial species explains the well known affinity of bacterial protoplasm for the basic stains, and limits its reaction with acid dyes. This does not mean, however, that acid dyes do not deserve a place in staining techniques. They react with the basic groups of the cell under the proper conditions, and, in fact, acid fuchsin, fast green, aniline blue, orseilline, etc., have been employed with marked success especially when used in phenolic solutions acidified with acetic acid. Acid dyes, moreover, present over basic dyes the advantage of giving better differentiation and of showing less tendency to stain debris and especially mucous material (which probably consists in many cases of acidic polysaccharides). It is worth noting that, whereas it had been empirically discovered that the staining efficacy of basic dyes is improved by the addition of alkali (alkaline methylene blue, etc.), the formulae for the use of acid dyes always call for the addition of acids to the staining system (Conn and Holmes, 1928; Maneval, 1941).

The selective staining of the enteric group on lactose eosin methylene blue agar provides an interesting illustration of the influence of pH on the uptake of dyes by bacteria. It appears probable that the production of acid by lactose fermenters lowers the pH sufficiently to allow eosin to be taken up by the individual cells, whereas this acid dye is not taken up at the normally alkaline reaction of the medium. The occasional blue colonies which are observed on E M B agar are those forming enough alkali to produce a pH high enough to cause dissociation of the eosin methylene blue complex and staining with methylene blue (Wynne, Rode, and Hayward, 1942).

Other Factors Which Affect the Staining Reaction.—Although the effect of pH on the staining process can be interpreted by assuming that the reaction between dyes and bacteria obeys the law of mass action, it must not be ignored that other phenomena certainly complicate the results. At very acid reactions, for instance, basic dyes are converted from normal dye salts to polyacidic dye salts, and become less colloidal. Under the same conditions, acid dyes pass, on the contrary, from normal dye salts to acid dye salts and to free color acids which are more colloidal than the acid dyes. These changes in colloidal properties probably modify and complicate the mechanism of the staining process (Holmes, 1929).

The reagents used in the fixation technique can also modify the staining characters of the preparation. Thus, formaldehyde, by combining with the amino groups, renders proteins more acidic and increases their affinity for basic dyes. On the contrary, polyvalent heavy metals (Hg^{++} , Al^{+++} , Cu^{++} , etc.) combine with the carboxyl groups of amino acids and thus move their iso-electric points to higher values (Tolstouhov, 1928).

Finally, as will be emphasized further in the discussion of the mechanism of the Gram technique, the interpretation of the reactions which occur between the cell and the dye is affected by the methods of decolorization used during staining. The ideal decolorizer should introduce no ionic complications; it should remove uncombined dye by solution only, and not by virtue of its own acidic or basic character. Acetone comes nearest to being chemically inert from this point of view, and has consequently been used as decolorizer in the determination of iso-electric points of bacteria (Stearn and Stearn, 1928a) (Chapter III:3)

Granted the multiplicity of factors which complicate the staining reaction, the following appears justified as a general statement of the problem. The bacterial substance is amphoteric in nature, and capable of combining chemically with basic dyes above its iso-electric point and with acid dyes below that point. This reaction is an ionic exchange, basic dyes displacing other cations adsorbed on the cell, whereas acid stains acting as anions have the opposite effect. The exchange tends to reach stoichiometrical pro-

portions and the dyes seem to compete with other ions for the same reactive groups of the cell. In the light of this hypothesis, the iso-electric values defined by the reactions of the cell with the dyes seem to indicate that the different bacterial families are characterized by chemical groups of different activities. Thus, as will be pointed out later in this chapter, the iso-electric points of Gram-negative organisms are clustered around pH 5-6, whereas those of the Gram-positive species reach pH 2-3. These values indicate that the various bacterial species are characterized by proteins with different numbers of acidic and basic groups, or that other acid radicals (phosphoric acid, sulfuric acid esters, amino sugars, etc.) are concerned in the reaction with the dye. We shall see that there is definite indication that ribonucleic acid plays a part in the Gram stain; in any case, it appears that a more thorough analysis of the mechanism of staining reactions may eventually serve as a guide in the detection of some of the reactive groups of the bacterial cell.

3. MECHANISM OF THE GRAM-STAINING TECHNIQUE

The Gram Technique.—The Gram-staining technique was developed as an empirical procedure for the differentiation of bacteria in tissues. It is now realized, however, that the behavior of microorganisms towards this procedure is correlated with so many other structural, chemical and biological differences, such as other staining characteristics, acid-base properties of the protoplasm, permeability of the cell membrane, resistance to toxic agents, toxicity of the bacterial bodies for animal tissues, etc., that the division between Gram-positive and Gram-negative species must correspond to some fundamental difference in cellular organization.

Notwithstanding variations in detail, the principle of the technique is as follows: the bacteria are first stained with a basic dye of the triphenylmethane group (crystal violet, for instance), at slightly alkaline reaction, and are then mordanted with iodine in solution in potassium iodide, or with other agents such as picric

acid. Subsequent washing of the stained preparation with water and with a neutral decolorizer (usually alcohol or acetone) removes the dye from the Gram-negative forms, whereas the Gram-positive remain fully stained. The difference is rendered more striking by counterstaining with another dye of a color contrasting with the one used in the initial staining; while the counterstaining fails to take on the Gram-positive organisms, it completely displaces whatever may be left of the initial stain on the Gram-negative cells.

Although many different theories have been offered to explain the mechanism of this complex process (Bach, 1930), we shall limit our discussion to those which have stimulated accurate observations concerning the factors involved in the outcome of the reaction.

Comparative Acid Base Properties of Gram-positive and Gram-negative Species.—When determinations are made of the pH range at which both acid and basic dyes are equally retained by bacterial cells, it is observed that the Gram-positive species have their "iso-electric" points clustered around pH 2.0, and the Gram-negative organisms around pH 5.0. Intermediate between the two groups are those organisms which are apparently variable to the Gram stain no matter how carefully the technique of staining is standardized, and which have been referred to as unstable Gram-positive bacteria (Churchman, 1927; Stearn and Stearn, 1925). A few examples of this generalization have already been presented in Figures 8 and 9, and more extensive data on the same subject appear in Tables 7 and 8 (Stearn and Stearn, 1931a).

The retention of dye in the Gram stain is determined by washing the stained bacteria with alcohol (or acetone) after they have been treated with iodine or some other mordant. If the so-called iso-electric point of the cellular material is the determinant factor in the outcome of the staining reaction, it is the iso-electric point of the mordanted cell which should condition its reaction with the basic dye. It has been pointed out in this respect that the compounds which have been suggested as substitutes for iodine in the Gram reaction all have in common the property of being mild

TABLE 7

ISO-ELECTRIC POINTS OF A NUMBER OF BACTERIAL CELLS NOT TREATED WITH IODINE

GRAM-POSITIVE ORGANISMS		GRAM-NEGATIVE ORGANISMS		GRAM-VARIABLE ORGANISMS	
Organism	pH of Iso-Electric Point	Organism	pH of Iso-Electric Point	Organism	pH of Iso-Electric Point
<i>B. cereus</i>	2.9	<i>B. coli</i> (communior)	5.0	<i>B. diphtheriae</i>	3.5-4.0
<i>B. subtilis</i>	1.9	<i>B. coli</i> (communis)	5.5	<i>B. dysenteriae</i> (Shiga)	3.2
<i>Micrococcus tetragenes</i>	2.9	<i>B. typhosus</i>	4.8	<i>B. proteus</i>	3.8
<i>Strep. anhemolyticus</i>	2.2	<i>B. aerogenes</i>	5.2	unknown bacillus (isolated from cancer tissue)	2.6
<i>Strep. pyogenes</i>	2.1	<i>B. aertrycke</i>	5.8		
<i>Staph aureus</i>	2.8				

Data from Stearn and Stearn (1931a, Table II, p. 451).

TABLE 8

ISO-ELECTRIC POINTS OF A NUMBER OF BACTERIAL CELLS FOLLOWING TREATMENT WITH IODINE

GRAM-POSITIVE ORGANISMS		GRAM-NEGATIVE ORGANISMS	
Organism	Apparent Iso-Electric Point pH	Organism	Apparent Iso-Electric Point pH
<i>B. cereus</i>	1.9	<i>B. coli</i> (communior)	4.4
<i>B. subtilis</i>	1.1	<i>B. coli</i> (communis)	4.9
<i>Micrococcus tetragenes</i>	1.8	<i>B. typhosus</i>	4.1
<i>Strep. anhemolyticus</i>	1.1	<i>B. aerogenes</i>	4.5
<i>Strep. pyogenes</i>	1.2	<i>B. aertrycke</i>	5.2
bacillus from cancer tissue	1.2	<i>B. proteus</i>	3.1
<i>B. diphtheriae</i>	2. to 3		

Data from Stearn and Stearn (1931a, Table IV, p. 455)

oxidizing agents (iodine, bromine, picric acid, trinitrobenzene, trinitrobenzoic acid, trinitrocresol, potassium dichromate, etc.): in fact, practically any mild oxidizing agent can give satisfactory results. These substances, by oxidizing the components of bacterial protoplasm, render the latter more acidic and lower the pH at which they are still capable of retaining the basic dye used in the Gram stain (Stearn and Stearn, 1924a).

If, on the other hand, picric acid, potassium dichromate, etc., shift the iso-electric points of all organisms in the same direction by rendering them more acidic, the advantage of mordanting in the Gram technique appears questionable. It has been found, however, that the shift toward lower iso-electric points caused by iodine and other oxidizing agents is definitely greater in the Gram-positive than in the Gram-negative organisms, so that the difference in acidic strength between the two groups is amplified by the mordanting process. The effect of iodine is specially noticeable on those organisms classified as "Gram variable," as may be seen in the results presented in Table 8 (Stearn and Stearn, 1931a).

The number of observations available is unfortunately too small to establish as a general law the differential effect of iodine (and other oxidizing agents) on the acid base properties of Gram-positive and Gram-negative species. If confirmed, however, the phenomenon transcends in importance its relation to the Gram stain, since it indicates a chemical difference between the two groups of organisms. Thus, it has been reported that the lipids extracted from Gram-positive differ from those extracted from the Gram-negative organisms, particularly because the former contain a much larger proportion of unsaturated acids which have a great affinity for iodine (Burdon, 1944; Jobling and Petersen, 1914; Williams, Bloor and Sandholzer, 1939). Whether the increased acidity resulting from oxidation of these unsaturated lipids accounts for the shift in iso-electric point of the cell as a result of mordanting in the Gram stain, remains an unexplored possibility.

Role of the Decolorizer.—If Gram-positive bacteria retain the

dye because of the ability of their cellular constituents to form with it a chemical complex which resists decolorization, the Gram reaction obviously depends upon the chemical activity of the decolorizer. Some decolorizers act, not only as neutral solvents of the dye, but also by competing with the bacterial substance by virtue of their own acid or basic properties. In fact, it has long been known that when amyl, propyl, butyl, ethyl, and methyl alcohols are compared with reference to their decolorizing ability, each one of these solvents decolorizes fewer bacterial strains than the one following. The difference in decolorizing ability also affects the rate of decolorization, and, for example, methanol decolorizes more rapidly than ethanol and propyl alcohol (Conn, 1928; Kiskalt, 1901). This relation suggests that the decolorizing efficacy of alcohols varies inversely with their molecular weight. It is also true, on the other hand, that acid strength increases in the same order as decolorizing efficacy, and differences in the latter can therefore be interpreted as due to a mass action competition between acid decolorizer and acid bacterial proteins. Indeed, it is found that whatever the organism considered, acidic decolorizers such as methyl alcohol, phenol, lysol, aldehydes, etc., decolorize all preparations stained by basic dyes, whereas basic decolorizers such as anilin, readily decolorize preparations stained with acid stains (Stearn and Stearn, 1928c, d).

The ideal decolorizer, then, is one which removes uncombined dye by solution only and not by its acid or basic properties; ethyl alcohol and acetone have such slightly acidic properties that they are adequate from this point of view. The fact that the iso-electric point of *E. coli* determined by observing retention of the dyes after acetone washing, corresponds also to the maximum buffering power as determined by titration, may be considered as a confirmation of this view (Stearn and Stearn, 1928c, d).

Effect of the Integrity of Cellular Structure on the Outcome of the Gram Stain.—The data which have just been presented establish a correlation between the acid base properties of the cellular material and its behavior toward the Gram technique. There are a number of facts, on the other hand, which indicate that the out-

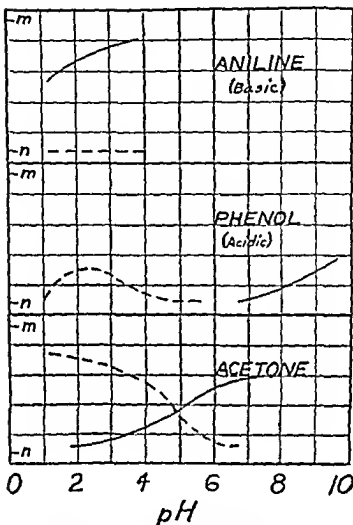


FIG. 10—Showing the apparent retaining power of *Escherichia coli* for gentian violet (unbroken lines) and acid fuchsin (broken lines) as a function of pH. Abscissae represent pH values, while ordinates give the intensity of retained color, after decolorization with the decolorizer indicated, based on an arbitrary scale. The scale lies between the ordinate m , representing maximum retained color, and n , representing complete decolorization. The acetone point lies at a pH-value of about 5, the phenol point is indeterminate since there was complete decolorization through the total pH-range between 5 and 7, the aniline point would have to be obtained by extrapolation and seems to lie close to pH 0, or even at a negative pH-value. (From Stearn and Stearn, 1923, fig. 2, p. 90.)

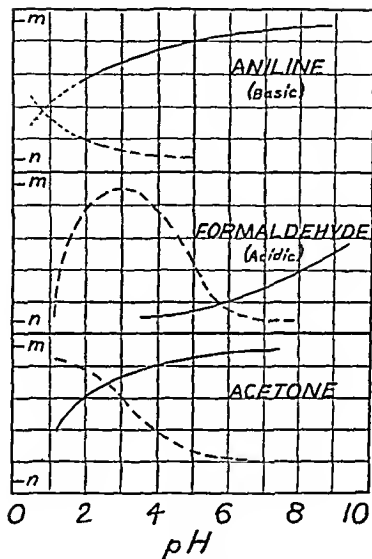


FIG. 11.—Showing the apparent retaining power of *Staphylococcus aureus* for gentian violet (unbroken lines) and acid fuchsin (broken lines) as a function of pH. Abscissae represent pH-values while ordinates give the intensity of retained color, after decolorization with the decolorizer indicated, based on an arbitrary scale. The scale is between the ordinates m , representing maximum retained color, and n , representing complete decolorization. The acetone point lies at a pH-value of about 2.6, the formaldehyde point at about 5.8, and the anilin point (obtained by extrapolation) at about 0.8. (From Stearn and Stearn, 1928, fig. 1, p. 89.)

come of the staining reaction is conditioned in some way by the structural integrity of the cell.

The cells of yeasts, staphylococci, pneumococci, streptococci, sporulating bacilli, etc., become Gram-negative when they are broken up by grinding in a ball mill or in a mortar, cut by microdissection, or more simply ruptured by gentle pressure between glass slides, whereas the morphologically intact cells in the same preparations remain Gram-positive. The protoplasmic contents exuded from the broken cell wall stain Gram-negative and the cell walls themselves remain practically unstained even when the staining technique is carried out at alkaline pH, *i.e.*, under conditions most favorable for the Gram-positive reaction (Benians, 1912-13; Burke and Barnes, 1929; Kemp, 1931; Schumacher, 1926).

There are other observations which also tend to show that Gram-positiveness depends upon the intactness of the cell wall. In the case of *B. mycoides*, the cells which show a sharp limiting wall when examined in the darkfield are also capable of retaining the Gram stain, whereas the abnormal cells in which the wall appears eroded are definitely Gram-negative (Stapp and Zycha, 1931). Pneumococci or staphylococci maintained for a short time at slightly acid reactions (pH 5.0) become Gram-negative when again transferred to a neutral medium. This change in staining reaction is due to the action of an enzyme which probably alters the cell wall although it does not cause dissolution or disintegration of the bacteria. Pneumococci killed by heat, formalin, phenol, etc., become Gram-negative under the action of this enzyme; on the contrary, treatment with crystalline trypsin leaves the cells in the Gram-positive state even after proteolytic digestion has destroyed a large percentage of the cellular proteins (Dubos, 1937c).

It must be mentioned at this time that several authors regard the Gram reaction as a phenomenon of colloid chemistry, and claim that the morphological integrity of the cell is required for the maintenance of surfaces available for adsorption of the dye. It has also been claimed that Gram-positive and Gram-negative

species differ quantitatively in adsorption power, perhaps because of difference in lipid content, and that the addition of iodine to gentian violet serves to change the physical properties of the dye and to render it more susceptible to adsorption by the cellular structures (Habs, 1932; Holmes, 1929).

Differences in Permeability between Gram-positive and Gram-negative Species.—The addition of iodine to methyl violet gives rise to the formation of a colloidal complex which is insoluble in aqueous media. Although both iodine and the dye can individually readily penetrate the cell wall, it is possible that the complex which they form is of such molecular dimension that it cannot escape from the Gram-positive organisms, whereas the Gram-negative species are permeable to it.

Like iodine, both potassium dichromate and potassium permanganate give a heavy precipitate with methyl violet. Alcohol dissolves the precipitate formed by the dichromate, and this substance can replace iodine as a mordant in the Gram technique, staining yeast Gram-positive and *E. coli* Gram-negative. On the contrary, the precipitate formed by potassium permanganate is only slightly soluble in the decolorizer, and when it is used instead of iodine, both Gram-positive and Gram-negative organisms resist decolorization and remain yellow-brown. These results have led to the hypothesis that the outcome of the Gram reaction depends, not upon the oxidizing property of the mordant, but rather upon the formation of a precipitate between the dye and the mordant which is independent of the protoplasm. This precipitate, although insoluble in water, must be soluble in the decolorizer, and of such a dimension as to pass through the cell wall of the Gram-negative but not of the Gram-positive species (Burke and Barnes, 1929).

The fact that the dye-iodine complex readily dissociates into its components when in solution in alcohol, presents difficulties to the adoption of this hypothesis. If both iodine and dye can penetrate the cell wall in the initial phase of the staining technique, they should be able to come out when the complex is allowed to dissociate during decolorization with alcohol. It has been observed, on the other hand, that the complex is only one-tenth as soluble as the

dye alone, so that the problem becomes one of equilibrium between associated and dissociated compounds. If it were possible to prevent the escape of iodine from the cell interior, the escape of the dye would be reduced at the same time. In other words, the property of Gram-positiveness could be explained if the Gram-positive species were found to possess a low permeability to iodine in alcoholic solution (Kaplan and Kaplan, 1933; Stearn and Stearn, 1930c).

To test cellular permeability to iodine, preparations stained by the Gram technique were treated with solutions of iodine in methyl alcohol. Although this solvent normally decolorizes the Gram-positive as well as the Gram-negative species, probably on account of its acidic character, the addition of small amounts of iodine to the alcohol is sufficient to rob it of its decolorizing power. The concentrations of iodine necessary to prevent dye escape vary with the different organisms, the highest concentrations being required for the Gram-negative species, while as little as 0.01 per cent iodine in methyl alcohol is sufficient to check the escape of the dye in some of the Gram-positive strains (Kaplan and Goldberg, 1934; Kaplan and Kaplan, 1933). It is questionable whether this technique gives a real measure of permeability to iodine.

Role of Ribonucleic Acid in the Gram Reaction.—It has often been observed that, within one given bacterial culture, some cells can be Gram-positive and others Gram-negative, and that the former appear larger than the latter, even when they all have been subjected to the same staining conditions (Churchman, 1927). The suggestion that the Gram-positive forms contain in the surface layer a material responsible for the staining reaction appears to have experimental confirmation from recent findings.

It is possible, by extraction with bile salts, to strip off the Gram-positive outer layer from a number of bacterial species, and to separate a Gram-negative skeleton which retains the shape of the organism, and a soluble fraction which is also Gram-negative. Even more important is the fact that the Gram-positive character can be restored by "replating" the extract back on the Gram-negative skeleton. Although the extract contains carbohydrates,

proteins, and magnesium ribonucleate, it is the latter substance which is significant in the staining reaction. In fact, it is possible to restore to the Gram-positive state, organisms rendered Gram-negative as a result of bile salt treatment, by placing them in a neutral solution of the magnesium salt of pure yeast nucleic acid. Organisms which are normally Gram-negative are not modified by this treatment, and, on the other hand, desoxyribonucleic acid cannot replace the ribo compound (Henry and Stacey, 1943).

These experiments suggest that the Gram-positive character resides in a protein-ribonucleate complex which exists at the cell surface. It is interesting, therefore, that one can bring about the change from the Gram-positive to the Gram-negative state by treating killed organisms with preparations of the enzyme ribonuclease. Pneumococci, streptococci, staphylococci, lactobacilli, sporulating bacilli, clostridia, and yeast have been shown to be susceptible to this treatment. On the other hand, the fact that ribonuclease is inactive against the living cells of the same species indicates that, as a result of death, the bonds by which magnesium ribonucleate is combined with the proteins and other constituents of the Gram-positive bacteria become more susceptible to the enzyme, or more readily reached by it (Bartholomew and Umbréit, 1944, and private communication; Dubos, 1937a; Dubos and MacLeod, 1938; Dubos and Thompson, 1938).

Other Staining Properties Correlated with the Gram Reaction.

—There exists a fundamental contradiction between the theories which consider the Gram stain as a simple acid base reaction between dye and cell protoplasm, and those which explain it in terms of some peculiar structure or property of the cell envelopes. In an attempt to reconcile these conflicting points of view, it has been suggested that the phenomenon studied by the proponents of the chemical (acid-base) theory is not the true Gram stain. The pseudo-Gram reaction with which they deal involves only the dye and protoplasm and occurs as readily outside as inside the cell. It does not give as heavy, intense a stain as the true Gram technique; and it results in such feeble coloration of the protoplasm that the counterstain is able to displace, or to mask, the original dye when

the staining is carried out on preparations in which the cells have been disrupted. The proponents of the chemical theory of the Gram stain have generally carried out their observations without using counterstains, and have consequently failed to recognize the essential difference between the two phenomena. The true Gram stain, involving both intact cell and the use of mordants, gives rise to such intense staining that the counterstain has no effect on the Gram-positive species (Burke and Barnes, 1929).

If the pseudo and the true Gram reaction are really different in their mechanisms, it is even more interesting that the classification of bacteria to which they give rise is essentially the same. As already mentioned, there are many other properties of microorganisms which are correlated with their behavior toward the Gram technique. Thus, the facts that Gram-positive cells are generally resistant to dissolution by alkalis and proteolytic enzymes, whereas Gram-negative are readily transformed into a jelly mass at slight alkaline reactions and are disintegrated by trypsin, obviously reflect some essential differences in the chemical constitution of the cell wall or of the whole cell body (Benecke, 1912, p. 112; Ryu, 1940). Of special interest for the present discussion are those techniques which seem to demonstrate, by direct staining reactions, the existence in Gram-positive species of a cell cortex absent in Gram-negative organisms. If one treats a preparation of Gram-positive organisms with tannin and then stains with almost any basic dye, the "ectoplasm" alone will take the stain. When, on the contrary, Gram-negative organisms are similarly treated, the whole cellular body is uniformly stained, either slightly or deeply, according to the species, the dye, and the time of treatment. When Gram-positive organisms are stained with a basic dye, then treated with tannin, and finally counterstained with another basic dye of contrasting color, the body of the cell stains with the first dye and the "ectoplasm" with the second. When Gram-negative organisms are similarly treated, they stain, either deeply or lightly, with the second color only (Gutstein, 1925, 1926; Maneval, 1929). These observations confirm the difference in properties between the cell envelopes of the two groups of microorganisms. In view of the

role of nucleic acid in the Gram reaction, it would be interesting to determine the effect of ribonuclease treatment on the outcome of the differential staining techniques which have just been outlined.

Quantitative Nature of the Differences Detected by the Gram Stain.—Although we have emphasized the contrasting behavior of Gram-positive and Gram-negative species under a variety of conditions, there is some suggestion that the differences between the two bacterial groups are of a quantitative rather than of a qualitative nature. Young cells are more likely to be frankly Gram-positive than are the older individuals in the same culture, a fact probably correlated with the greater affinity for the basic dyes exhibited by the younger organisms. Starvation of bacterial cultures results in a progressive decrease of Gram-positiveness and similarly the stainability of yeast cells is decreased when they are depleted of their ribonucleic acid content by mere washing at slightly alkaline reaction (Delaporte, 1939, p. 449; Stearn and Stearn, 1929b). Not only do individual cells within a culture vary in the intensity of their reaction to the Gram stain, but also there have been reports that the Gram behavior can be altered as a result of a sort of mutation. Furthermore, within one given bacterial group, the staphylococci or streptococci for example, different strains exhibit great variations in the intensity of staining; indeed, certain strains remain "Gram-variable" whatever the technique employed.

We have seen that the protoplasmic material of the typical Gram-positive is more acidic than that of the typical Gram-negative species, and that intermediate values often characterize the Gram-variable forms. There is also some indication that permeability to iodine varies progressively from one group to another. Thus, both the "chemical theory" and the "permeability theory" are compatible with the view that microorganisms can be arranged in a continuous series with reference to their behavior toward the Gram stain, and it will be shown later that the quantitative differences in staining properties are correlated with quantitative differences in susceptibility to the bacteriostatic action of cationic

antiseptics (Chapter VIII: 3). As pointed out in the general analysis of the mechanism of staining (Chapter III: 2), the wide range of iso-electric values exhibited by the various bacterial families suggests the participation of several different types of acidic groups in the reactions between the cells and the basic dyes. The understanding of the mechanism of the Gram stain, and of the differential susceptibilities of Gram-positive and Gram-negative organisms to various antiseptics, undoubtedly depends upon a more accurate knowledge of the nature, properties and distribution of the acidic groups of the bacterial cells.

4. STAINING PROPERTIES OF MYCOBACTERIA

Correlation Between Lipid Content of Mycobacteria and Their Staining Characteristics.—The property of acid fastness—or resistance to acid decolorization—is associated not only with resistance to decolorization by alcohols and alkalis, but also with poor stainability by ordinary techniques. In the Ziehl-Neelsen technique, for example, the staining of tubercle bacilli requires the use of a concentrated aqueous solution of fuchsin containing 5 per cent carbolic acid, for a few minutes at high temperatures, or for a longer time at lower temperatures. The acid fast forms of mycobacteria so stained retain the dye even after washing with alcohol containing 5 per cent of inorganic acid, whereas nonacid fast cells are completely decolorized under the same conditions. It is clear, therefore, that the value of the Ziehl-Neelsen technique depends upon two apparently independent properties; its intense staining power which permits clear definition of the acid fast forms, and the differential decolorization achieved by acid alcohol.

With the exception of a few strains of diphtheroid bacilli and actinomyces, of the bacterial endospores, and of some special animal cells, the property of acid fastness is peculiar to a group of organisms, the mycobacteria, which are also remarkable by the fact that they produce large amounts of lipids which accumulate in the bacterial bodies. The impression that acid fastness is in

some way associated with the fatty and waxy nature of the micro-organisms is indeed substantiated by a few experimental facts. Thus, conditions unfavorable to the synthesis of lipids are often unfavorable to the development of acid fastness. Conversely, it is possible by raising the glycerol content of Long's synthetic me-

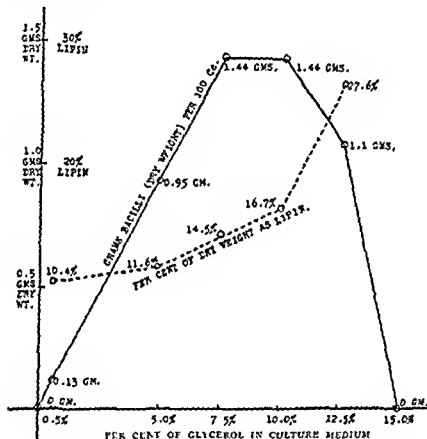


FIG. 12—The effect of glycerol on the lipid content, growth and acid fastness of the tubercle bacillus (see text).

dium from 0.5 to 12.5 per cent to increase simultaneously the total yield of bacteria, the lipid content in terms of per cent weight, and the intensity of acid fastness. When stained by the Ziehl-Neelsen technique, bacilli grown on 0.5 per cent glycerol appear rose-pink, while those grown on 10 to 12.5 per cent glycerol stain deep crimson; intermediate staining can be seen in cells grown in media containing from 1 to 5 per cent glycerol (Long and Finner, 1927).

Attempts to demonstrate that the lipids themselves constitute the acid fast material led to the discovery that most of the fats and waxes extracted by lipid solvents are not capable of retaining the dye after acid treatment. This property is displayed only by a certain fraction, first designated as mycol, which has now been isolated from the unsaponifiable wax in the form of an acid alcohol containing free hydroxyl groups and known as "mycolic acid." There is some evidence that the presence of free hydroxyl groups in the mycolic acid molecule is of significance in the property of acid fastness. Recent findings suggest that this substance exists in the cell in combination with a polysaccharide, and that the complex also possesses acid fast properties (Anderson 1929, 1940; Choucroun, 1939, 1940, 1943). It may be mentioned at this time that, although acid fast microorganisms are also Gram-positive, and although the two staining properties are destroyed simultaneously by a variety of treatments, there is no indication that they depend upon the same mechanism. Mycolic acid, in any case, does not retain the Gram stain (Kretschmer, 1934).

The apparent parallelism between acid fastness and lipid content, and the marked hydrophobic properties of mycobacteria, led early to the assumption that these organisms are surrounded by a waxy capsule which determines poor stainability and resistance to decolorization. As indicated in Chapter II: 4 microscopic and cytochemical methods fail to provide evidence for the existence of such a structure and, moreover, the hydrophobic character is also possessed by organisms which are not acid fast. Finally, prolonged extraction of tubercle bacilli with various fat solvents—xylene, petroleum ether, sulfuric ether, chloroform, acetone, and mixture of these solvents—removes large amounts of free lipids from the organisms, but does not affect their acid fastness as long as the extraction is carried out without excess heat and at neutral reactions (Anderson, 1940; Fethke, 1938; Knaysi, 1929; Macheboeuf and Fethke, 1934; Pfannenstiel, 1922; Wells and Long, 1932, p. 61). It appears, therefore, that although mycobacteria are characterized by the accumulation of readily extractable lipids, their peculiar staining properties are not explained thereby.

Acid Fastness and Integrity of Cellular Structure.—The existence in tubercle bacilli of a well-defined component, mycolic acid, which retains fuchsin in the Ziehl-Neelsen technique, even after it has been isolated from the cell in the form of a pure substance, appears to account satisfactorily for the acid fastness of these organisms. There are many facts, however, which suggest that the problem is in reality much more complex.

It has been repeatedly shown that mere mechanical disintegration of the bacilli by grinding them in an agate mortar or in a ball mill, or by crushing an unstained preparation between glass slides, is sufficient to destroy acid fastness. These simple mechanical treatments leave an amorphous mass of cellular debris which still stains with aniline dyes, but which no longer resists decolorization by acid alcohol. In other words, it is possible to destroy acid fastness by mere physical means, whereas prolonged extraction of the intact cells with lipid solvents fails to achieve the same end. A similar result can be obtained by causing the organisms to undergo autolysis even though the latter process does not destroy mycolic acid (Baisden and Yegian, 1943; Benians, 1912-13; Koch, 1897; Sherman, 1913; Wells and Long, 1932, p. 132).

We have emphasized that acid fast bacilli completely exhausted of their "free" lipids by thorough extraction with neutral organic solvents remain acid fast. It must be pointed out, however, that this technique of extraction does not remove all the lipids present in the cell. If bacilli first extracted in the cold and at neutrality are subsequently treated with boiling alcohol or with hydrochloric acid, they lose their acid fastness and release a further yield of 1 to 8 per cent of their dry weight as additional lipids. The time required to achieve liberation of these "bound lipids" is conditioned by the strength of acid used. At pH 1.3, for instance, only 5 minutes' boiling is sufficient to destroy acid fastness, whereas 5 days are necessary at pH 6.0; rapid action begins at pH 4.0. Results similar to those obtained with hydrochloric acid can be obtained with halogenated organic compounds (Anderson, 1929, 1940; Aronson, 1898, 1910; Boissevain, 1927; Bulloch and Macleod, 1904; Fethke, 1938; Long, 1922; Macheboeuf, Dieryck,

and Stoop, 1935; Macheboeuf and Fethke, 1934; Salimbeni, 1912; Wells and Long, 1932, p. 122).

It appears in summary that the acid fastness of mycobacteria is conditioned by the morphological integrity of some cellular structure which can be destroyed by mechanical, enzymic, or chemical means under conditions which do not destroy mycolic acid. In order to account fully for the mechanism of the Ziehl-Neelsen technique, it is therefore necessary to reconcile a number of well established facts. 1. There exists in the tubercle bacillus a hydroxy acid, mycolic acid, which exhibits acid fastness, even after it has been isolated from the bacterial cell as a pure substance. 2. Thorough treatment of the cell with neutral fat solvents fails to destroy acid fastness although it extracts large amounts of free lipids. 3. Disintegration of cell structure by mechanical, enzymic, or chemical techniques, destroys acid fastness without destroying mycolic acid. 4. Following acid treatment adequate to destroy acid fastness, an additional amount of lipids ("bound lipids") become available for extraction by neutral fat solvents.

It has been suggested that the resistance of the bound lipids to extraction by neutral solvents is due to the fact that, in the intact cell, they exist as a firm lipo-protein complex which is responsible for the peculiar permeability properties of acid fast bacilli. Hydrolysis of this complex by hydrochloric acid would simultaneously render the lipids free and extractable, and destroy acid fastness (Long, 1922). The fact that lipo-protein complexes have been recognized in other microorganisms and can be split by mild acid hydrolysis, is in favor of this hypothesis. It is also certain, on the other hand, that acid hydrolysis can, and certainly does, affect a great variety of other cellular structures, the destruction of which might allow passage of the lipids, free or bound, which are present inside the cell.

We must now consider the conflicting facts that mere mechanical disintegration of the cell destroys its acid fastness, whereas free mycolic acid displays resistance to acid decolorization. It must be noted in this respect that the amounts of mycolic acid which are required to demonstrate the acid fastness of this sub-

stance in the free state are greatly in excess of those present in the bacteria in a microscopic preparation. It appears worth considering that the integrity of the cellular structure is essential to concentrate and to organize in a particular arrangement within the cell the acid fast substance upon which the dye becomes adsorbed and from which it can be extracted only slowly. There are indeed several observations which indicate the importance of adsorption phenomena in the Ziehl-Neelsen technique. Thus, carbolic acid does not appear to act merely as a mordant in this technique; it forms with fuchsin a fairly well defined complex, an addition product, which is stable to boiling and which can be crystallized from water. This phenol-dye complex possesses peculiar colloidal properties which allow the tubercle bacillus to adsorb it more readily and to hold it more firmly (Holmes, 1929; Holmes and Hann, 1928).

The following facts also suggest that staining by the Ziehl-Neelsen technique involves a distribution of the dye on some cellular surfaces. Tubercle bacilli stained in the absence of electrolytes appear as uniformly stained rods, whereas the addition of electrolytes to the system causes a beaded appearance due to the accumulation of the dye in granules which are separated by unstained or poorly stained bands. Treatment with ethyl alcohol is sufficient to restore to a beaded preparation the appearance of uniform solid staining, and it is even possible to observe directly in wet preparations, under the microscope, the reversible passage of uniform to beaded staining under the influence of electrolytes and alcohol (Yegian and Baisden, 1942; Yegian and Budd, 1943).

The possession of mycolic acid is not a necessary condition of acid fastness. For example, the endospores of bacteria, *Taenia* eggs, human and animal hair, etc., retain carbof-fuchsin when treated by the Ziehl-Neelsen technique, although there is no evidence that these structures contain large amounts of lipids or of any substance related to mycolic acid. One might assume that, in these cases, resistance to acid decolorization depends upon mechanisms which are entirely different from the one which con-

ditions the staining properties of acid fast bacilli. The possibility remains, on the other hand, that acid fastness is not the unique attribute of a substance or group of substances, but is rather the result of some peculiarity in cellular organization related, for instance, to the physico-chemical property of the cell wall.

IV

ANALYSIS OF CELLULAR STRUCTURE BY BIOCHEMICAL AND BIOLOGICAL METHODS

Only such substances can be anchored at any particular part of the organism which fit into the molecule of the recipient combination as a piece of mosaic fits into a certain pattern.

PAUL EURLICH

1. ENZYMES AND CYTOLOGY

M*orphological Organization of Enzymes Within the Cell.*—The chemical events within the cell must be highly coordinated in order to permit an orderly progression of the various metabolic steps. It is certain therefore that the living cell is not merely a "bag of enzymes" lying freely about the cytoplasm, but that, on the contrary, the different enzymes are linked in some way with cellular structure and exist in the form of a well defined morphological arrangement of the biochemical units (Barron, 1943; Nilsson and Alm, 1940).

Only few specific facts can be quoted to illustrate the existence of this biochemical architecture. Great differences of optimum pH exist between the enzymic activities of intact cells and soluble cell-free preparations, a fact which suggests that, in the living organism, some enzymes are protected by membranes or other structures against changes in the reaction of the environment. In yeast, the pH activity curve of glucose oxidation exhibits such a wide optimal range that the enzyme seems to be unaffected by the reaction of the medium; the pH activity curve of trehalose oxidation, on the contrary, shows a very sharp optimum suggesting that the enzyme concerned is located outside of, or directly on,

TABLE 9

PH OF OPTIMUM ACTIVITY FOR AMINO-ACID DECARBOXYLASES

SUBSTRATE	ORGANISM	CELL-FREE ENZYME	INTACT CELL	Diff.
Histidine	<i>C. watchii</i>	4.5-5.0	2.5-3.0	2.0
	<i>E. coli</i>	5.5	4.0-4.75	0.75-1.5
Arginine	<i>E. coli</i>	5.5	4.0-4.75	0.75-1.5
Lysine	<i>E. coli</i>	6.0	4.5-5.0	1.0-1.5
Tyrosine	<i>S. faecalis</i>	5.5	5.0-5.5	0.0-0.5
Acetoacetic acid	<i>C. acetobutylicum</i>	5.5	4.0-5.0	0.5-1.5

Data from Gale (1943, table 2, p. 166).

the plasma membrane. It has been assumed for similar reasons that the enzyme lactase is present on or outside the membrane, whereas the fermentation enzymes in general seem to be situated within the cell (Gale, 1943; Myrbäck and Vasseur, 1943). The course of respiratory processes in *Escherichia coli* makes it likely that the relation between dehydrogenase and oxygenase (Atmungsferment) is one of intimate juxtaposition at some intracellular surface, and a similar morphological association has been claimed for coenzyme linked reactions between the dehydrogenase systems (Cook, Haldane, and Mapson, 1931; Dewan and Green, 1937).

Cytolysis usually results in a marked decrease of enzymic activity. It is probable that, in many cases, the inactivation of the dehydrogenases which accompanies cellular disruption is due to the "washing out" of the coenzymes and to their dilution in the surrounding medium. However, the decrease in activity of the glucose dehydrogenase of *E. coli* caused by freezing and thawing, and the loss of the glucose and lactic dehydrogenases of *Micrococcus lysodeikticus* caused by lysis with lysozyme, cannot be corrected by the addition of an excess of coenzyme to the system. In these instances, therefore, enzyme inactivation is not due merely to the dilution of the cell contents, but more likely to the actual destruction of the enzymes themselves which appear to be

linked in some way with the cellular structure (Yudkin, 1937a).

The examples which have just been quoted are only suggestive of the morphological arrangement of the intracellular enzymes. More direct evidence of their location will come from the development of specific histochemical techniques such as the one which has revealed the situation of phosphatase in the kidney cells (Lipmann, 1941).

The Phenomena of Autolysis.—Autolysis is a complex phenomenon which involves cellular disintegration accompanied by a variety of enzymic processes affecting the cellular proteins, lipids, nucleic acids, polysaccharides, etc. In the living cell, the intracellular enzymes are separated from their respective substrates by membranes which probably require energy for maintenance. These membranes break down after death, allowing a variety of enzymic reactions, which are held in abeyance during life and the sum total of which constitutes autolysis. It is possible, on the other hand, that there exists one particular enzyme capable of attacking a special morphological component essential to the maintenance of cell structure and which, because it initiates the disintegration process, deserves more specifically the designation of autolytic enzyme. In any case, autolysis, when allowed to run its course unchecked, involves so many simultaneous reactions that little can be learned of its nature and mechanism by the mere analysis of the end products. A better understanding of the essential primary process would be facilitated if it were possible to prevent secondary reactions and to identify the initial step which is responsible for cellular disintegration.

Pneumococci are notorious for the ease with which they undergo autolysis. They rapidly lose their morphological integrity and staining characteristics when resuspended in saline solutions, and this initial injury is followed by a slower enzymic destruction of the cellular constituents. If living pneumococci are precipitated by large volumes of cold acetone or alcohol, then dried with ether, one obtains a powder which, when resuspended in boiling water, iodine, or formol solution (media which inhibit the autolytic enzymes) consists of typical Gram-positive cocci. If, however, the

bacterial powder is resuspended in a neutral aqueous medium at 30 to 40° C., a "flash lysis" occurs within a few seconds, resulting in a complete dissolution of the cell bodies which is caused by the autolytic enzymes (Dubos, 1937b).

Rapid lysis can also be obtained by adding to the culture or cell suspension small amounts of surface-depressing and other toxic substances (bile, Na desoxycholate, lauryl sulfonate, chloroform, etc.), and in this case again, the lytic process does not take place in the absence of enzyme action. The lytic action of surface active substances is probably due to a combination of several properties. These substances rapidly kill the cell without inactivating the enzymes concerned in the primary steps of autolysis, and they peptize the products of cellular disintegration. Dissolution does not require extensive proteolysis or lipolysis. Indeed, these enzymatic processes can be completely inhibited by dissolving pneumococci in large concentrations of sodium desoxycholate, but even in this case, dissolution of the bacteria does not take place if conditions preclude enzymic action (Atkin, 1926; Dubos, 1937b; Goebel and Avery, 1929; Neufeld and Etinger-Tulczynska, 1930; Sturdza, 1938).

Pneumococci killed with formaldehyde remain morphologically intact and Gram-positive as long as an excess of the inhibitor is present in the system. As soon as the inhibitor is removed, however, there begins a slow enzymic process which causes the cells to become Gram-negative without changing their gross morphology. A similar effect can be obtained by killing pneumococci with acetic acid at pH 4.5. Neutralization of the preparation initiates a limited enzymic process which releases in solution a ribonucleohistone, and which changes the cells from the Gram-positive to the Gram-negative state. It appears, therefore, that the whole autolytic system of pneumococci can be inhibited by formaldehyde and acetic acid, but that a certain component of this system, the one responsible for the change from the Gram-positive to the Gram-negative state, is only reversibly inhibited, and recovers its activity following removal of the inhibitor (Dubos, 1937b, c, d, 1938a; Dubos and Thompson, 1938).

The enzyme system responsible for the autolysis of pneumococci can be obtained in solution and can then transform pneumococci killed by heat, formol, iodine; etc., into a mass of Gram-negative debris. This action is not due merely to nonspecific proteases, lipases, etc., since the same enzyme system, very active against pneumococci, is entirely inactive against other microbial species (with the exception of *Streptococcus viridans*, a bacterial species closely related to the pneumococcus). The autolytic system must contain, therefore, certain enzymes which possess specificity for some cellular structures of the pneumococcus. Although the autolytic complex undoubtedly consists of a mixture of many different enzymes, the particular one which changes the bacteria from the Gram-positive to the Gram-negative state can remain active under conditions where the nonspecific proteolytic, lipolytic, saccharolytic, etc., activities are completely inhibited or greatly minimized (Avery and Cullen, 1923; Dubos, 1937c; Meyer, Dubos and Smyth, 1937; Wollman, 1932).

Autolytic systems comparable to the one which has been recognized in pneumococci also exist in other bacterial species. For example, tubercle bacilli, killed and desiccated with alcohol ether, and resuspended in neutral aqueous media, undergo an autolytic process which results in loss of acid fastness (Baisden and Yegian, 1943). A more rapid action of the same order can be obtained by merely killing the organisms with toluid and, as in the case of pneumococci, it is possible to arrange the experimental conditions so that loss of staining property, unaccompanied by cellular lysis or disintegration, is the only gross evidence of autolytic action.

It is tempting to believe that the cell substrates which are altered during these controlled lytic reactions are the same upon which depends the retention of the dye in the Gram and Ziehl-Neelsen techniques (Chapter III:3, 4). Unfortunately, no accurate information is available concerning the chemical structure of the substrates attacked by the primary autolytic enzymes. Progress in this direction would undoubtedly contribute to the understanding of cellular organization, with particular reference to

structures which are of importance for the pathogenic behavior of bacteria.

Enzymes as Cytological Reagents.—Ideally, cytological techniques should disclose not only the morphology, but also the chemical composition of cellular objects. It is to this end that cytologists are always striving to convert staining and microscopic procedures into microchemical reactions. Thus, the Feulgen technique makes use of Schiff's reaction for the detection of the aldehyde group of desoxyribose; photography in certain wave lengths of the ultraviolet range permits the identification of purine and pyrimidine bases; osmic acid and fat soluble dyes stain selectively certain fatty substances, etc. Unfortunately, few cytological reactions possess sufficient specificity to give chemical definition to the objects which they reveal.

There are several properties of enzymes which give them great potential value as reagents capable of increasing the range and specificity of cytochemical techniques. Although few enzymes, if any, possess absolute specificity, the action of each one of them is localized to a fairly well defined class of substances, to a definite type of chemical linkage. Furthermore, enzymes function at reactions which are little removed from physiological pH, and they can be used, therefore, where other more drastic reagents would cause gross alterations of delicate cellular structures. Finally, techniques are available for the discovery and preparation of enzymes selectively adapted to the performance of almost any type of biochemical reaction. It can be predicted, therefore, that either used singly or in combination with other procedures, enzymes will come to play an important part in the analysis of biological problems in general, and of cellular structure in particular (Dubos, 1939). Many types of bacteria are susceptible to the action of certain enzymes present in tissues and particularly in leucocytes. In fact, the bactericidal and bacteriolytic properties exhibited by tissues and cell extracts, and the immune bacteriolysis mediated by complement, probably require the participation of enzymes (Wells, 1925).

Leucocytes, pancreas extract, and many other tissues contain

a heat resistant enzyme which transforms heat killed pneumococci and other bacteria from the Gram-positive to the Gram-negative state. Preparations of this heat resistant enzyme can hydrolyze ribonucleic acid; and, moreover, crystalline ribonuclease can also destroy the Gram-positive property (Bartholomew and Umbreit, 1944, and personal communication; Dubos, 1937a; Dubos and MacLeod, 1938; Dubos and Thompson, 1938). Although the relation between ribonuclease activity and effect on staining characteristics thus appears convincing, it must be kept in mind that there exist in animal tissues other enzymes possessing physico-chemical characters similar to those of ribonuclease, and which can also affect staining properties. Lysozyme is one of these enzymes; since it is heat resistant and exhibits the same solubility properties as ribonuclease, it may contaminate even crystalline preparations of the latter and complicate the study of its effects on the susceptible bacterial cells.

The addition of minute amounts of egg white to suspensions of living cells of certain microbial species (*Micrococcus lysodeikticus* or *Sarcina lutea*, for example) brings about the rapid dissolution of these organisms. This action is caused by a heat stable lytic agent, lysozyme, which is also present in a number of other tissues and fluids of animal and plant origin. There is, in fact, suggestive evidence of the existence of a number of different principles which exhibit lysozyme-like activity, but which differ in their physico-chemical properties and in the range of organisms which are susceptible to them (Fleming, 1922, 1932; Mesrobian and Noepfel, 1938; Thompson, 1940; Thompson and Gallardo, 1941).

Lysozyme is an enzyme which, like ribonuclease, is resistant to heat at acid reactions, and which has been isolated in the form of a basic protein of low molecular weight (Abraham, 1939; Meyer, Thompson, Palmer, and Khorazo, 1936). It depolymerizes and hydrolyzes a mucoid (acetyl aminopolysaccharide) which occurs in animal tissues and in susceptible bacterial cells (Epstein and Chain, 1940; Hallauer, 1929; Meyer, Palmer, Thompson, and Khorazo, 1936). When successive photographic records are made of the course of lysis by lysozyme, the bacterial cells are seen to

become more and more transparent, without undergoing complete disintegration (Boasson, 1938). It appears, therefore, that the lytic process consists in an increase of cellular permeability which permits the cell material to diffuse into the medium, and which is due to the alteration of a polysaccharide contained in one of the cell envelopes. The discovery that a certain bacterium is susceptible to lysozyme can consequently be used as evidence that this organism possesses as an essential component of its structure the substrate, probably an acetyl amino polysaccharide, which is hydrolyzed by the enzyme.

There are many other situations where the use of enzymes has revealed important facts of bacterial cytology. Thus we have mentioned above in Chapter II:4 that certain enzymes can destroy the capsules of pneumococci and of streptococci, and that trypsin can inactivate the specific M substance of group A hemolytic streptococci without affecting the viability of the cells so treated. The readiness with which these substances can be hydrolyzed without affecting viability suggests that they are superficially located, and that they are more in the nature of excretion products than true structural constituents of the cell. Other examples will be given later in this chapter to illustrate the possibility of analyzing cellular structure by the combined use of enzymic and immunological methods.

2. IMMUNOLOGICAL REACTIONS AND CELLULAR STRUCTURE

Antibodies as Specific Reagents.—The immune reaction elicited by the injection of one type of bacterial cells, living or dead, is not a simple phenomenon. Bacteria are complex organisms, made up of a multiplicity of chemical constituents, and many of these are capable of eliciting the production of specific antibodies. It is not surprising, therefore, that the injection of one type of bacterial cells usually results in the production of several antibodies, each directed against one particular cellular component.

These different constituents obviously bear a definite spatial morphological relationship to each other in the intact cell. Some

are masked by membranes and become exposed only as a result of cellular disintegration; others are peripherally disposed and in direct contact with the environment. From the immunological point of view, this stratification of the cellular structures is important on at least two grounds. It undoubtedly affects the immune reaction of the injected host, and determines the type and amount of the antibodies produced; it conditions also the response of the bacterial cell to a given antibody, since the intact microorganism reacts much more readily, if not solely, with the antibodies which are directed against those of its constituents exposed at or near the surface.

Attempts to reconstitute cellular architecture from the study of the reactions between bacterial antigens and specific antibodies have been helped by the readiness with which bacteria undergo variation. The chemical composition of any given bacterial species is not a constant, permanent property. Cultures undergoing variation give rise to mutant forms which are deficient in one or more of the antigenic components present in other phases of the same culture, and immunization of animals with a variant form deficient in one of the antigenic components elicits the production of a serum deficient in the corresponding antibody.

Instead of using deficient mutants as antigens, it is often possible to inactivate selectively (by heat or chemical means, for instance) one or several of the antigenic components of a given culture, and thus to obtain less complex antigens for immunization. Even more specific reactions can be obtained, of course, by immunizing animals with the substances isolated in pure form from the bacterial cell. Finally, by absorbing sera with selected antigens, one can remove at will any one of the corresponding antibodies and obtain reagents exhibiting a narrower range of activity and therefore greater selectivity.

Serological methods can thus be utilized as micro-analytical tools, especially in view of the fact that, because of their specificity and great reactivity, antibodies can detect small amounts of the homologous antigens in the presence of large quantities of contaminating substances. The skillful use of these immunological

procedures has contributed much to the understanding of cellular structure. In principle, the method involves the preparation of antibodies specific for each one of the chemical constituents of the cell, the use of these antibodies as specific reagents for the detection and preparation in pure form of these cellular constituents, and finally the interpretation of antigen-antibody reactions in an attempt to define the relative positions occupied by these components in the living cell.

Flagellar Antigens.—Although the salmonella are, in general, motile, flagellated organisms, it was discovered very early that motile cultures of the hog cholera bacillus give rise to nonmotile variants (Smith and Reagh, 1903). Sera prepared against the motile strains agglutinate both the motile and nonmotile cultures, but exhibit a much higher titer against the former than against the latter; furthermore, the clumps given by agglutination of the motile strain are fluffy and form rapidly, while the clumps given by the nonmotile strains are tight, granular, and form much more slowly. On the contrary, sera prepared against the nonmotile strains agglutinate both strains to the same degree, giving in each case the slow, granular type of agglutination. Sera prepared against the motile strains, when absorbed with the nonmotile strains, lose the ability to agglutinate the nonmotile bacilli, but still agglutinate the motile strains, whereas sera prepared against the nonmotile strains lose their agglutinating power for both strains when absorbed with the motile strains. It appears from these facts that the immunological behavior of the motile hog cholera bacillus can be analyzed in terms of two independent antigenic constituents, one present in the flagella, the other in the cell body. The nonmotile variant has lost the flagellar antigen but retains the somatic constituent.

The difference between the flagellar and somatic types of agglutination has now been recognized in a great many other bacterial species, and has been particularly well studied in the case of the strain of the proteus bacillus used for the serological diagnosis of typhus fever. Whereas the flagellated form produces on agar a thin-spreading growth which has been described by the



German expression "Hauch," the nonflagellated variant grows in isolated colonies and has been called the "Ohne Hauch" form. The abbreviations H and O have now become associated with the antigenic constituents, flagellar and somatic, which determine the immunological behavior of the two bacterial forms (Weil and Felix, 1917).

It is necessary to mention at this time that the flagella of one given culture can exist in several immunologically different forms and give rise to the phenomenon which has been described under the name of phase variation of the H antigens (Andrewes, 1922). When a subculture from a young colony of a biphasic salmonella (*i.e.*, one capable of producing two different H antigens) is incubated, all the flagella in the culture are antigenically identical for a few hours, but thereafter both flagellar antigens are found in the culture. It was believed at first that the antigens of one phase were characteristic of the specific culture type under consideration, while the other phase was present in all biphasic salmonellas. The terms "specific" and "nonspecific" flagellar phases were introduced to describe this situation, but in reality the antigens of either phase occur in various types although it is true that the "specific" antigens are generally restricted to a smaller number of types (Chapters II:5 and V:4).

The flagellar antigens of the different Gram-negative bacilli—both in the specific and nonspecific phases—exhibit marked phenomena of cross agglutination in the corresponding antisera. In order to account for these cross serological reactions, it has been assumed that each phase of each bacterial strain can possess several antigens, some of which also occur in other strains. Because of the large number of strains and of the complexity of their antigenic structure, the salmonella are now usually described by formulae which symbolize their somatic and flagellar antigens. In the Kauffmann-White classification, the somatic O antigens (to be considered later) are represented by roman numerals, whereas the flagellar antigens of the specific phase are designated by small letters, and those of the nonspecific phase by arabic numerals. When the alphabet was exhausted the flagellar antigens were

further designated Z_1 , Z_2 , etc. Thus the symbols I, II, XII, a and IX, XII, d, which stand respectively for *S. paratyphi* A and *E. typhosa*, reveal that these two cultures are monophasic with reference to their H antigens, whereas the formula VI, VII, c, 1, 5 (*S. paratyphi* C) corresponds to a diphasic form (Bornstein, 1943; Kauffmann, 1937, 1941b).

Occurrence of the O Antigens in Gram-negative Bacilli.—We have mentioned the existence in the virulent Gram-negative bacilli of the somatic antigens O which are responsible for the slow granular agglutination exhibited by these organisms when mixed with the homologous antisera. These somatic O antigens are usually resistant to heat and to proteolytic enzymes, and therefore differ completely from the H antigens not only in immunological specificity, but also in chemical nature.

The different types of Gram-negative bacilli can be readily differentiated on the basis of their O antigens by the titers of agglutination in the corresponding antisera. On the other hand, these organisms also exhibit marked cross agglutination in the same sera, due to immunological relationships between their somatic O antigens. As in the case of the flagellar constituents, it can be assumed that there exist a number of different O antigens (which in the Kauffmann-White Schema are symbolized by roman numbers) which occur in different combinations, each characteristic of a given particular culture. Thus, *S. paratyphi* A contains antigens I, II, XII, and *S. paratyphi* B contains antigens I, IV, V, XII; the common possession of antigen XII determines cross serological relationship, whereas the other antigens peculiar to each type determine its specificity.

The repeated occurrence of the same antigenic components in the different bacterial types is naturally of great importance for their classification and for the understanding of their phylogeny. It also raises the question of the nature of the relationship which the different specific somatic antigens bear to each other within a given culture. Are these antigens present in different cells which, in a given culture, occur in a definite proportion characteristic of the strain? Are they simultaneously present as separate independ-

ent structural and chemical entities in each and every cell? Or do the O specificities correspond to several immunological determinant groups of a single antigenic molecule? It is probably relevant that the quantitative relation between the several antigens of a given salmonella type appears to be the same for all the strains which contain them. Thus, the factors IV and V of *S. paratyphi* B occur in all strains of this particular type in the same quantitative ratio. Furthermore, the two factors are precipitated together by a serum containing only the anti-IV, as well as by a serum containing only the anti-V antibody. It appears, therefore, that the two antigenic specificities are bound together in a single unit and that they are part of a complex molecule possessing multiple antigenic determinants (Meyer, 1938a; 1939).

Chemical Nature of the O Antigens.—It is possible to extract from the smooth variants of Gram-negative organisms a polysaccharide fraction which precipitates in high dilution in the homologous antiserum prepared with the whole bacterial cell, and which possesses the immunological specificity of the strain used for immunization. When prepared by the use of drastic chemical methods, especially at strongly alkaline reactions, these polysaccharides can no longer elicit in rabbits the production of antibodies, although they remain capable of reacting in specific antisera. In other words, they behave as haptens, but not as complete antigens (Furth and Landsteiner, 1928, 1929; Landsteiner and Levine, 1927; Morgan, 1931; White, 1931).

There have been devised a number of methods which release the specific polysaccharide in a fully antigenic form. These methods include extraction of the cells with trichloroacetic acid (Boivin, Mesrobian, and Mesrobian, 1933; Mesrobian, 1936), with diethylene glycol (Morgan, 1937), with phenol (Morgan and Partridge, 1941; Palmer and Gerlough, 1940), with urea (Walker, 1940), aqueous pyridine (Perlman, Binkley and Goebel, 1944), digestion with trypsin (Raistrick and Topley, 1934), shaking with chloroform (Morgan, 1941), etc. All these procedures result in the insolubilization, denaturation, or destruction of the largest percentage of the cellular proteins and release in solution a phos-

pholipid-protein-polysaccharide complex which is toxic and antigenic. Dialysis of this soluble fraction followed by fractional precipitation with alcohol or acetone yields a product which represents 5 to 20 per cent of the weight of the bacilli, and which accounts for the largest part of their toxicity and antigenic properties. The complete antigens thus obtained are of high molecular weight—8,000,000 in one specific example—but the fact that both the sedimented and nonsedimented fractions obtained by the ultracentrifugation of the antigen of *E. typhosa* are antigenic and toxic indicates that the state of aggregation of the substance can vary without affecting the biological activity (Boivin, 1939a; Kahler, Shear, and Hartwell, 1943).

The complete antigens can be further dissociated into their component parts by a number of techniques which vary in efficacy from one organism to another. In a very general way, it appears that treatment with formamide permits the removal of the phospholipid component and leaves a protein-polysaccharide unit which is toxic and antigenic. Treatment with aqueous solution of phenol results in denaturation of the protein component, leaving a large molecular size polysaccharide which gives extremely viscous solutions. On the other hand, hydrolysis of the protein-polysaccharide complex with acetic acid releases the protein in the native form, but depolymerizes the polysaccharide to a point where it no longer gives viscous solution, although it still precipitates specifically in homologous antiserum (Morgan and Partridge, 1940, 1941, 1942; Partridge and Morgan, 1942).

Following separation from the protein, the free polysaccharide is no longer antigenic in rabbits and loses much if not all of its toxicity. When, however, the native viscous form of this substance is added to a solution of the native form of the protein under the proper conditions, there is formed a molecular complex of definite composition which is precipitable with alcohol, and which possesses the toxic and antigenic properties of the material originally extracted from the cell. Similar proteins obtained by the same technique from different organisms (*salmonella* and *shigella*) can also combine with the native polysaccharide, and moreover, dif-

TABLE 10

COMPONENTS OF THE PURIFIED O ANTIGENS OF *E. typhosa*
OBTAINED BY ACID HYDROLYSIS
(Morgan and Partridge)

FRACTION	PER CENT OF TOTAL EXTRACT	CHEMICAL NATURE	SEROLOGICAL NATURE
1. ether soluble	5-7	phospholipid	not antigenic
2. acid insoluble, soluble in dilute alkaline solution	20	conjugated protein, previously referred to as polypeptide-like material	strongly antigenic, not specific (practically identical with protein from <i>Shigella dysenteriae</i>)
3. water soluble	50-55	degraded polysaccharide	non-toxic hapten

Data of Morgan and Partridge, quoted by Bornstein (1943, Table 5, p. 465).

ferent polysaccharides (even of plant origin, like agar or gum arabic) can combine with the native bacterial proteins (Partridge and Morgan, 1940).

Titration curves indicate that combination with the polysaccharide increases the base binding capacity of the protein in a manner similar to the effect of formaldehyde (Freeman, 1943). Further studies of these reactions should yield interesting facts concerning the chemical properties of the complete antigens of Gram-negative bacilli and of the significance of these substances in cellular organization.

Although the knowledge concerning the O antigens which has been outlined in the preceding pages has been derived chiefly from the study of salmonella and shigella cultures, it appears probable that the same general pattern of antigenic structure will be found valid for other Gram-negative bacilli and perhaps cocci. The immunochemical analysis of the brucella organisms illustrates this view.

The smooth variants of *Brucella melitensis*, *suis*, and *abortus* can be differentiated by the titer of agglutination in homologous antisera, but they also exhibit cross immunological relationships

TABLE II

PRECIPITATION AND AGGLUTINATION REACTIONS OF RABBIT IMMUNE SERA PREPARED AGAINST DIFFERENT ARTIFICIAL COMPLEXES

MATERIAL USED AS ANTIGEN	NUMBER OF DOSES AND AMOUNT	PRECIPITATION TEST DILUTIONS OF POLYSACCHARIDE	AGGLUTINATION TEST* DILUTIONS OF IMMUNE SERUM										
			1:10 ⁴	1:10 ³	1:10 ²	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	
Complex prepared in formalin from high viscosity polysaccharide and "Shiga" polypeptide	3 × .05 mg.	$\left\{ \begin{array}{l} 4 \\ 4 \\ 3 \end{array} \right.$	$\left\{ \begin{array}{l} 4 \\ 4 \\ 4 \end{array} \right.$	$\left\{ \begin{array}{l} 2 \\ 2 \\ 1 \end{array} \right.$	4	4	4	4	4	3	2	1	
Complex prepared from high viscosity polysaccharide and "Shiga" polypeptide in aqueous solution	3 × .05 mg.	$\left\{ \begin{array}{l} 3 \\ 3 \\ 3 \end{array} \right.$	$\left\{ \begin{array}{l} 4 \\ 4 \\ 4 \end{array} \right.$	$\left\{ \begin{array}{l} 2 \\ 2 \\ 2 \end{array} \right.$	4	4	4	3	0	0	0	0	
Complex prepared from low viscosity polysaccharide and "Shiga" polypeptide by treatment in formalin	3 × .05 mg. + 3 × .01 mg	$\left\{ \begin{array}{l} 0 \\ 0 \\ 0 \end{array} \right.$	$\left\{ \begin{array}{l} 0 \\ 0 \\ 0 \end{array} \right.$	$\left\{ \begin{array}{l} 0 \\ 0 \\ 0 \end{array} \right.$	0	0	0	0	0	—	—	—	
High viscosity "Shiga" polysaccharide adsorbed on α -lactalbumin particles	3 × .05 mg †	$\left\{ \begin{array}{l} 0 \\ 0 \\ 0 \end{array} \right.$	$\left\{ \begin{array}{l} 0 \\ 0 \\ 0 \end{array} \right.$	$\left\{ \begin{array}{l} 0 \\ 0 \\ 0 \end{array} \right.$	0	0	0	0	0	—	—	—	
High viscosity "Shiga" polysaccharide adsorbed upon denatured rabbit serum proteins	3 × .05 mg †	$\left\{ \begin{array}{l} 0 \\ 0 \\ 0 \end{array} \right.$	$\left\{ \begin{array}{l} 0 \\ 0 \\ 0 \end{array} \right.$	$\left\{ \begin{array}{l} 0 \\ 0 \\ 0 \end{array} \right.$	1	0	0	0	0	—	—	—	

* Formalized whole culture of "smooth" *Bact. shiga*

† Calculated as specific polysaccharide

Data from Pattinige, S. M. and Morgan, W. J. J. (1910, Table IV, p. 187)

which suggested the existence in these organisms of two antigens, A and M, present in different amounts in the surface of the three bacterial types (Miles, 1939; Wilson and Miles, 1932). However, all attempts to separate the postulated A and M antigens have failed and all the purified fractions so far tested have been found to possess the two types of immunological activities. The soluble antigenic and toxic materials obtained from the different brucella by extraction with trichloroacetic acid consist of phospholipid-polysaccharide-protein complexes similar to those obtained from other Gram-negative bacilli, and they exhibit the same cross reactions as the intact organisms (Lisbonne and Monnier, 1936; Pennell and Huddleson, 1937, 1938; Pop, Damboviceanu, Barber, and Marinov, 1938).

Quantitative analysis of the precipitin reactions given by these soluble antigens with the homologous and heterologous antisera obtained by immunization with bacterial suspensions, reveals that the homologous reaction behaves as a single antigen-antibody system, whereas the cross reactions follow a different quantitative course similar to the one found for most cross reactions previously studied. It appears, therefore, that the hypothesis of two antigens (A and M), varying in amounts in the different varieties of salmonella, is an oversimplification, and that *B. abortus*, *suis*, and *melitensis* contain closely related but not identical antigens which cross react to a marked extent (Pennell and Huddleson, 1938).

When brucella organisms are extracted with 2 per cent phenol or with chloroform water, there goes in solution a fraction which, after precipitation with ammonium sulfate and sedimentation at 14,000 rpm, is obtained as a toxic antigenic complex (PLAPS); it exhibits marked anisotropy of flow and it contains not only the A and M antigens but also the antigenic components of the rough (R) phase. The complex PLAPS can be dissociated by the addition of sodium dodecyl sulfate to a simpler form PLAPS, and loses then its milky appearance and anisotropy of flow. Extraction of the complex with alcohol ether containing 0.5 per cent HCl removes a phospholipid (PL) but does not affect

serological activity. An arginine containing protein (S) can be removed by further treatment with acetic acid also without loss of precipitating power. The material (AP) is still antigenic and toxic and is similar to the preparations obtained by trichloroacetic acid extraction. Acid hydrolysis destroys the biological properties of AP and liberates another phospholipid, free phosphate, formic acid and free amino groups.

Although the position of (PLAPS) in the bacterial cell is still uncertain, the study of the S \rightarrow R variation in *Br. melitensis*, and of the associated changes in agglutination reactions, indicates that the progressive loss of smooth antigen is accompanied by a gradual uncovering of an R antigenic surface. It appears, therefore, "that (PLAPS) is predominant in the surface of the bacterium in the form of a shell or capsule, in which PLAPS units are built in such a way that the shell of (PLAPS) disrupts into plate or rod-like pieces. The yield of (PLAPS) from *Br. melitensis*, a cocco-bacillus $0.6 \times 1.6 \mu$, has never exceeded 10 per cent of the dry weight of the organism. If we assume that the bacterial mass and (PLAPS) are hydrated to the same extent, it is easy to calculate that a surface layer of (PLAPS) one-tenth of the bacterial weight would be 1-20 $m\mu$ thick. The errors in any of these assumptions have but little effect on this figure. It is clear that a capsule of this thickness would not be detectable microscopically. This is not evidence that (PLAPS) is in the form of a capsule, but that if it were so, it would not be visible." (Miles and Pirie, 1939).

The Vi Antigens.—Cultures of the typhoid bacillus known to possess the classical O antigen may fail to agglutinate in sera containing the O antibody. This inagglutinability is correlated with the presence of another cellular component which has been called the Vi antigen because it is present in the typhoid cultures isolated from human pathological material and appears therefore to be related to virulence. It is possible to obtain anti Vi sera free of O antibodies by immunizing animals with culture variants containing the Vi but not the O constituent, or by adsorbing sera containing both antibodies with cells containing the

O but not the Vi antigen. These specific Vi antisera precipitate the Vi substance and agglutinate Vi cells, whether or not the latter contain the O antigen; they also protect experimental animals against infection with Vi cultures (Almon, 1943; Felix and Pitt, 1934, 1935). The Vi substance is rapidly released in solution when the bacilli are suspended in physiological NaCl solution, but not in CaCl_2 solution (Kisida, 1941). Moreover, the Vi antigen is extremely labile to heat, dilute acid, alcohol, phenol, etc., although its ability to react in antisera can often survive treatments which destroy its antigenicity (Felix, 1941; Felix and Bhatnagar, 1935; Felix and Petrie, 1938; Felix and Pitt, 1936) (Chapter VII:4).

Its lack of stability appears, therefore, to differentiate the Vi from the O antigens. It must be remembered, on the other hand, that all O antigens are not equally stable, and that, for example, antigen V of the salmonella is fairly susceptible to heat, to acid, and to alkali (Kauffmann, 1936a; Landsteiner and Furth, 1928). Neither are the differences exhibited by the O and Vi components of the typhoid bacillus with reference to their precipitability by uranyl salts, their iso-electric agglutination points, and their electrophoretic migration, sufficient to establish that these antigens belong to different classes of substances (Boivin, and Mesrobianu, 1938C; Combiesco and Soru, 1939; Ogonuki, 1940; Rouchdi, 1938; Topley *et al.*, 1937). It is true that the O and Vi antigens of the typhoid bacillus can vary independently of each other, but so can the different O antigens in other Gram-negative species (Boivin, Izard, and Sarciron, 1939; Kauffmann, 1935, 1937, 1941b; Meyer, 1938b). Nor is it established that Vi is more important in determining virulence than the O antigen, and furthermore, the possession of Vi is not a unique property of virulent strains of *Eberthella typhosa*, but can also be recognized in a number of other, nonpathogenic species (Almon, 1943; Bornstein, 1942; Luippold, 1942, 1944; Stuart, Wheeler, Rustigian, and Zimmerman, 1943). Except for the fact that the presence of Vi decreases the agglutinability of typhoid bacilli in O antiserum, it is difficult to find at the present time any sharp

differential character to set this antigen apart from the other somatic specific antigenic components of the Gram-negative bacilli. The fact to be reported later in this chapter that the agglutination corresponding to the Vi-anti Vi reaction appears to differ in character from that due to O-anti O may ultimately serve as a guide for a more accurate definition of the structural significance of the Vi antigen.

Other Antigens of Gram-negative Bacilli.—We have seen that the production by Gram-negative bacilli of variant forms deficient in one or several of their antigenic components has greatly facilitated the recognition and isolation of the H, O and Vi antigens. Bacterial variability is not limited to these immunologically well defined factors, but affects also many other cellular components. Although the differences in biological properties, immunological behavior and chemical composition which are associated with these other types of variation are often obscure, some are worth brief mention at the present time, since they illustrate the potential value of the phenomena of variation for the study of cellular structure.

Cultures of shigella and salmonella can grow in the M (mucoid) form under a variety of experimental conditions, and the M forms are often poorly agglutinable in O antisera unless they are previously boiled (and thus probably freed of the mucoid material). In contrast to the O polysaccharide, the M substance of salmonella does not appear to be type specific, and antisera produced with mucoid variants agglutinate all live M cultures (Fletcher, 1918; Kauffmann, 1936a; Morgan and Beckwith, 1939; Müller, 1910; Trawinski, 1923). The M substance is a polysaccharide resistant to heat; its relation to the bacterial capsule has been considered in Chapter II:4. It may be mentioned at this time that there has been recognized by immunological reactions an agglutinin which is common to certain strains of lactose and nonlactose fermenting coliform bacilli, which like Vi can inhibit O agglutination, but which is distinct from all other known antigens. This agglutinin is not associated with virulence and is widely distributed among Gram-negative bacilli;

it can be lost in subculture and thus lead to the development of specific variants (Stamp and Stone, 1944).

Although the S \rightarrow R variation was first recognized by changes in colonial and cellular morphology, by increased spontaneous agglutination of cell suspension, and by decrease of virulence, it is now more commonly defined in terms of loss of the specific O polysaccharide antigen (Arkwright, 1920, 1921, 1924). The variation from the typical S to the typical R form undoubtedly involves many complex phenomena which have not been fully elucidated, and, for example, the loss of the O antigen can at times be observed without being accompanied by complete change of the morphological characters or spontaneous agglutinability. In any case, it is certain that the R forms of the salmonella exhibit little or no serological specificity. When R cultures are treated chemically by the methods which have been mentioned for the separation of the O (polysaccharide) antigens from the S forms, one obtains small amounts of a fraction somewhat similar in chemical properties to the complete specific antigen, but which is devoid of serological specificity and which exhibits, in fact, considerable cross reactions not only with other salmonella but also with the shigella group (Waaler, 1935; White, 1929). Although preparations of the R antigen so far obtained are rich in polysaccharide, the chemical nature of the substance which is responsible for the serological character of the R variants is not established (Boivin, 1939b; Henderson, 1939b; Mackenzie and Pike, 1939; Malek, 1938; White, 1931).

Old strains of salmonella kept for many years on artificial media are often found to possess neither the smooth O nor the rough antigen. They seem to correspond to a further step in the way of loss variation, and have been called the ρ variants. From these ρ forms, as well as from the R and S forms, one can obtain by extraction with 95 to 97 per cent alcohol, acidified with HCl, a protein which has been designated as Q. This protein is readily soluble in acid and alcohol, and in slightly acidic or basic aqueous media; it is, however, insoluble at neutrality in physiological salt solution, and probably plays a part in conditioning the insta-

bility of cell suspension in saline solutions. The Q protein is antigenic and gives rise to sera which agglutinate the ρ forms well, the R forms slowly, but not the S forms—although Q is also present in the latter (White, 1932).

After removing from the cell the O, R and Q antigens, one can still extract by 75 per cent acid alcohol another antigenic protein (T) which also gives rise to antisera capable of agglutinating the R and ρ , but not the S forms (White, 1933).

Finally, there should be mentioned another substance which can be extracted from the cell by alcohol and chloroform, and which is insoluble in acetone and aqueous media. This substance appears to be a phospholipid and has been recognized in both the S and R variants. Although it has not yet been shown to possess immunological activity, it contributes to rendering cell suspensions less stable by virtue of its hydrophobic properties (White, 1928).

Contrasting Antigenic Patterns of Different Bacterial Groups.—The data outlined in the preceding pages concern the antigenic structure of Gram-negative bacilli. It appears that the different members of this group, although differing in immunological specificities, are all characterized by a common pattern of organization of their cellular antigens; this pattern constitutes another criterium of differentiation of the Gram-negative from the Gram-positive species (Doivin and Mesrobianu, 1938b). Since the present survey does not aim at a complete description of immunological knowledge, only two groups of organisms, the streptococci and pneumococci, will be selected to illustrate the immunochemical characteristics of the Gram-positive species.

Streptococci can be divided into a few large groups characterized by the possession of somatic polysaccharides "C" which are serologically different for each group. These polysaccharides have been obtained in solution and purified by extracting ground defatted bacilli with acetate buffers or with hot formamide, and by fractionating at varying pH. They appear to be chemically bound with the nucleoproteins and to account in part for the serological specificity of the latter. The nucleoproteins themselves

constitute a complex fraction which can be separated into several components by adequate techniques. Like the C carbohydrates, these proteins appear to be internal constituents of the cell (Heidelberger and Kendall, 1931, 1936; Lancefield, 1928, 1933, 1941; Mudd and Wiener, 1942; Zittle and Harris, 1942).

Each one of the groups defined in immunological terms by the C polysaccharides can be further subdivided into types characterized by specific antigens. Because of their importance in human pathology the hemolytic streptococci of group A have been most thoroughly studied from this point of view, and type differentiation in this group has been shown to be due to two different types of antigenic constituents, the M and T substances, which vary from type to type.

Although T has been obtained in solution and purified by isoelectric precipitation at pH 2.5, and by its ability to resist the action of pepsin and trypsin, nothing is known as yet of its chemical nature. Available evidence indicates that it is present at the cell surface (Lancefield, 1940, 1941; Lancefield *et al.*, 1943, 1944, and personal communication).

The M substances have been isolated from the matt variants of group A streptococci; they are present only in traces, if at all, in the glossy variants. They have been separated from the cell by extraction with dilute HCl and can be obtained as a homogeneous protein, free of phosphorus, with a molecular weight of 41,000. The M proteins are readily hydrolyzed by proteolytic enzymes. The fact that trypsin, acting on living streptococci, destroys their immunological specificity without affecting their viability, suggests that the M substance is located at the surface of the cell (Hirst and Lancefield, 1939; Lancefield, 1928, 1940, 1943; Pappenheimer, Williams, and Zittle, 1942; Zittle, 1939, 1942).

In addition to hyaluronic acid, the high molecular polysaccharide present as a capsule in the mucoid phase (Chapter II:4), other cellular components of streptococci have been recognized by taking advantage of their ability to elicit the production of antibodies which can be used as specific reagents (Lancefield,

1941). Thus, as in the case of Gram-negative bacilli, there is slowly emerging from purely immunochemical analysis a composite picture of the many components of the streptococcal cell. Similar progress could be described for a number of other bacterial species and a special section will be devoted later in the present chapter to the analysis of the structure of pneumococci by immunological and enzymic techniques (Chapter IV:3). In the following pages, an attempt will be made to outline the possible applications of immunochemical methods to the understanding of cellular architecture and to the study of the phylogenetic relationships among bacteria.

Immunochemical Analysis and Cellular Architecture.—The detection, isolation and identification of cellular components is only one of the tasks of the cytologist and immunochemist. The relative position of these components in cellular architecture is of special interest to the student of infection, since the role played by the different bacterial substances in pathogenicity is conditioned, not only by their chemical nature and biological properties, but also by the readiness with which they come in contact with the tissues of the invaded host. It appears worth while therefore to illustrate how cytological and immunological reactions have been used to define the location in the intact living cell of the different substances recognized by immunochemical methods.

The problem is simplest in the case of the H antigens. That these substances are constituents of the flagella is indicated by their absence in nonflagellated variants and by the fact that, in the presence of H antiserum, motile forms lose their mobility, modify their flagellar structure, or even lose the ability to produce flagella. Direct microscopic examination in strong light reveals that the H agglutination of typhoid bacilli consists in an immobilization of the flagella which later become entangled (Pijper, 1938, 1941a) (fig. 13). Electron microscope pictures of bacteria sensitized by anti-flagellar antibodies also show that the flagella appear thickened. The antibody film measured by comparing the thickness of sensitized and non-sensitized flagella

corresponds to a film of globulin molecules radially disposed (Mudd and Anderson, 1941) (fig. 15).

Knowledge of the position of the other antigenic components in the cell has been gained by more indirect methods. In general terms, these methods consist in observing the readiness with which the specific antibodies react with the corresponding antigens in the intact cell. Since antibodies are globulins and therefore of large molecular size, it is unlikely that they can readily penetrate the plasma membrane. It is assumed, therefore, that if an antibody reacts readily with a given living cell, causing, for instance, its agglutination or lysis, or protecting animals against infection with it, the homologous antigen with which it combines is a surface constituent of this particular microorganism. If, on the contrary, the antibody fails to react with the intact cell, although it can combine with one of its components after disruption of the cellular structure, then this component is assumed to be located within the cell and to be masked by other more superficial constituents. Thus, the fact that the Vi antibody agglutinates cells containing the Vi antigen and protects animals against experimental infection with Vi organisms indicates the existence of this antigen at the cell surface. The same observation and conclusions hold true for the O antigens. Furthermore, the fact that the presence of Vi prevents the agglutination of O cells by anti O serum, has been interpreted to mean that Vi is a more superficial component than O (Almon, 1943).

It will be recalled also, that, in the salmonella, the antibody directed against the Q substance agglutinates the ρ variants very rapidly, the R cells only slowly, and the S forms not at all, and that anti Q does not protect experimental animals against infection. On this basis, it has been concluded that the Q protein is a surface constituent of the ρ variant, which becomes partly or completely masked by other more superficial components (the R and O antigens) in the R and S forms. Similar observations and deductions have led to the view that the T substance and certain nucleoproteins and carbohydrates of the salmonella are situated even more deeply inside the cell (White, 1932, 1933).

It is worth mentioning in this respect that whereas the R cells of salmonella treated with Milson's reagent immediately give a strong protein test, the S cells remain Millon negative under the same conditions, and become positive only after removal of the soluble specific substance. This observation provides additional evidence for the conclusion that, in the S forms, the cellular proteins are separated from the environment by a nonprotein layer, consisting probably of the specific polysaccharide (White, 1929a).

Perhaps more convincing, because more direct, information has been yielded by microscopic studies of the reaction between cell and antibody. Thus, darkfield cinematography of typhoid agglutination shows that "in O agglutination the bacteria are brought together by a definite force which acts along the long axis of their bodies, and this force is powerful enough to overcome the natural repulsion of the bacterial bodies. The direction of the force causes the O agglutination clumps to assume a definite crystalline appearance."

"In Vi agglutination an entirely different process was encountered. Here darkground microscopy showed that the first effect of the Vi agglutinins is a visible paresis of the motile organs of the typhoid bacilli, leading to slower and even erratic movement. The erratic movement makes the bacteria collide. As, however, the natural repulsion which is a feature of bacterial suspensions is still at work at the same time, collisions are not frequent. There is no question of any mutual attraction, which is such a striking phenomenon in O agglutination. In Vi agglutination the clumsy movements of the bacteria force them up against one another, as it were against their will.

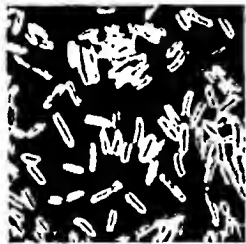
"The second feature of Vi agglutination is that the surface of the bacillary bodies develops a marked stickiness. Whenever the area of contact between two bacteria that have involuntarily collided is sufficiently extensive, this stickiness holds them together. If the contact is slight, the erratic motility is usually strong enough to make them separate again. The formation of clumps thus becomes an extremely slow and tedious process. For any linking up to become permanent, large areas of contact are

essential. Polar contacts are not stable, lengthwise contacts are. The stickiness may convert polar contacts into lengthwise contacts. It follows that the majority of the more stable clumpings that do come about show a parallel pattern, i.e. the bacteria are in contact along the greater part or the whole of their sides. The final pattern of small clumps thus resembles a brick wall, the individual bacteria representing the bricks." (Pijper, Crocker, and Todd, 1943.)

The Vi agglutination, therefore, appears as the only agglutination in the true sense of the word since it seems to be due, not to mutual attraction, but to a stickiness of the bacteria which causes them to attach themselves to each other side by side, thus confirming earlier evidence that the Vi antigen is the most superficial component.

It is clear that the information concerning cellular structure which is revealed by immunochemical analysis rests on such indirect evidence that it should be considered, at best, only as a first approximation. The outcome of the reaction between antigen and specific antibody does not depend only on the superficial versus internal situation of the antigen. It is conditioned by many other factors, known and unknown, among which can be mentioned the steric hindrance caused by neighboring groups of the cell and by the strength of the charge which they carry, the presence of hydrophobic and hydrophilic substances which affect the stability of the bacterial suspension, etc. Because of these limitations, no single fact is sufficient to warrant a conclusion concerning the morphological significance of any structure detected by immunological behavior. It is certain, however, that immunochemical methods constitute tools of great specificity and flexibility for the mapping of the cellular constituents into an orderly picture.

Immunological Specificity and Phylogenetic Relationships.—Bacteria can be separated into groups and subdivided into types by means of immunological reactions which reveal differences often not detectable by any other tests. It appears, therefore, that classification based on immunochemical specificity should be



a



b



c



d

FIG. 13.—Darkground studies of flagellar agglutination of *Eberthella typhosa*. a Normal unagglutinated typhoid bacilli in broth culture. b Early H-agglutination of typhoid bacilli. Note granules on tails of flagella producing thickening. c Early H-agglutination of typhoid bacilli. Note granules on tails of flagella producing thickening. d H-agglutination of typhoid bacilli showing thickening of both bacillary walls and flagellar structures. (Microphotographs and legends were obtained through the generosity of Dr. Adrianus Pijper, of Pretoria, South Africa. Methods described in Pijper, 1938, 1940, 1941.)

more reliable than that founded on fermentation reactions since antigenic structure is, on the whole, a more stable character than biochemical activity. On the other hand, there occur among all bacterial groups and types a number of cross serological reactions which present some analogy with the cross reactions observed between the blood cells of different animal species. As is well known, these phenomena have provided valuable information concerning the evolutionary relationships in the animal kingdom, and it is probable that immunochemical relationships among bacteria will eventually permit the reconstruction of phylogenetic lines of development (Boyd, 1943, p. 137).

The application of immunological reactions to bacterial taxonomy presents, however, several difficulties. Bacterial cells contain a multiplicity of antigenic components, each of which gives rise to an antibody endowed with a certain specificity. One or several of these antigens can be lost by the cell as a result of variation (Chapter V:4) and antigenic behavior is therefore conditioned by the dissociative phase in which the culture happens to be. To be of significance for the establishment of phylogenetic relationships, comparisons of antigenic structure must be made on cultures in the same dissociative phase, and, moreover, must refer to corresponding cellular antigens (flagellar, capsular, homologous somatic components, etc.).

Another difficulty arises from the frequent occurrence of immunochemical relationships which are undoubtedly accidental in nature. The specificity of the antigenic reaction depends upon the chemical structure of the antigenically determinant group of a given substance, and the chance occurrence of the same group in widely different species can thus give rise to cross reactions of no taxonomic significance. It is to such chemical accidents that is due the immunological relationships between type 11 pneumococci and certain strains of type B Friedländer bacilli (Avery, Heidelberger, and Goebel, 1925, Julianelle, 1926) (Table 12). There are many examples of cross reactions between pneumococci, yeast and fungi (Neill, 1939). It appears also from the results presented in Table 13 that growth of a certain strain of streptococcus

TABLE 12
AGGLUTINATION REACTIONS OF TWO STRAINS OF FRIEDLÄNDER'S BACILLUS AND PNEUMOCOCCUS TYPE II IN HOMOLOGOUS
AND HETEROLOGOUS SERA

CULTURE	IMMUNE SERA											
	Anti-Friedländer Sc.				Anti-Friedländer E				Antipneumococcus Type II			
	1:5	1:10	1:20	1:40	1:80	1:5	1:10	1:20	1:40	1:80	1:160	1:320
Friedländer's bacillus (Sc.)	+++	+++	+	-	-	-	+	+	+	+	+	+
Friedländer's bacillus (E)	-	-	-	-	-	+	+	+	+	+	+	+
Pneumococcus Type II.	-	-	-	-	-	+	+	+	+	+	+	+

The symbols +++ indicate complete, compact, disk-like agglutination; ++, marked, compact, disk-like agglutination; +, clumping, more easily broken up; -, partial clumping; ±, slight clumping; -, negative.

Data from Avery, Heidelberger, and Goebel (1925, table I, p. 712).

TABLE 13

COMPARISON OF REACTIONS OF STREPTOCOCCUS II "GROUTING" SERUM WITH THE REACTIONS OF TYPES 2 AND 20 ANTISTREPTOCOCCUS AND OF ANTILEUCONOTOC SERUMS

Antisera	SUPERNATANT FLUIDS OF CULTURES OF GROUP II STREPTOCOCCI				JICI-EXTRACT OF GROUP II STREPTOCOCCUS CELLS			
	Sucrose Cultures		Dextrose Cultures		Sucrose Cultures		Dextrose Cultures	
	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2
Streptococcus II (strain 1)	++	+	++	+	+++	++±	+++	++±
Leucococcine (strain A or B)	++++	++++	0	0	+++	++	0	0
Phocococcus type 2 or 20	0	0	0	0	0	0	0	0
" " 1 or 12								

0 = no precipitation with undiluted or diluted antigen

+, ++, +++ = precipitation with undiluted, 1:10, 1:100 and 1:1,000 dilutions of the antigens.

Data from Neill, Surz, Mehre, and Jaffe (1941, Table II, p. 342).

of group H in the presence of sucrose results in the production of a polysaccharide which precipitates in antileuconostoc and in types II and XX antipneumococcus immune sera (Neill, Sugg, Hehre, and Jaffe, 1941) (Table 13). Cross serological reactions also occur between anthrax bacilli, type XIV pneumococci and the group A human blood substance (Ivánovics, 1940b) (Table 14).

TABLE 14

HOMOLOGOUS AND HETEROLOGOUS SEROLOGICAL REACTIONS BETWEEN THE ANTHRAX BACILLUS, PNEUMOCOCCUS TYPE XIV AND GROUP A BLOOD SUBSTANCES

SERUM	AGGLUTINATION OF HUMAN RED CELLS	PRECIPITATION WITH DIFFERENT HAPTENES		
		Anthrax C	Pneumo- coccus XIV	Group A
Anthrax horse serum	A -	+	+	(+)
Anthrax rabbit serum	A -	+	-	-
Pneumococcus type XIV horse serum	A +, B +, O +	?	+	+
Pneumococcus type XIV rabbit serum	A -, B -, O -	-	+	-

(+) = after partial hydrolysis.
? = not tested.

Data from Ivánovics (1940, Table 6, p. 385).

The various flagellar (H) and somatic (O) antigens of the salmonella are widely distributed among other coliform bacilli, shigella, pasteurella, etc., and even pneumococci have been found to agglutinate in certain antisalmonella sera (Bornstein, 1943; Kauffmann, 1941a) (Table 15).

Granted the difficulties of interpretation which result from cross immunological reactions due to chemical accident, it remains probable that a critical scrutiny of immunochemical relationships can serve as a useful guide in many problems of bacterial taxonomy. Thus it is likely that, in the sporulating anaerobic

TABLE 15
SALMONELLA ANTIGENS IN NON-SALMONELLA BACTERIA

CLASSIFICATION OF ORGANISMS	ANTIGENS
<i>E. coli</i>	part of XII IV, XII IV, V, XII IV, XXVII, XII part of VI, VII I, 5 Vi, I, 5
Coliform bacteria, paracolon bacilli	the complete O antigen of <i>S. enteritidis</i> various combinations of O antigens, pre- dominantly I, VI and XIII I, II 2 ₁ , 2 ₁₂ 2 ₁ , 2 ₇ , 2 ₁₁ , 2 ₁₂
<i>Shigella paradyenteriae</i> Flexner (V)	VI, XIII
(<i>Shigella</i> B)	(Related to K of <i>Salmonella</i>)
<i>Pasteurella pseudotuberculosis</i>	part of the O antigen of group B
<i>Pasteurella avicula</i>	antigenic extract related to VI
<i>Pneumococcus</i> types 35, 35 A, 35 B	part of the O antigen of <i>S. Linker</i>

Data from Bornstein (1943, Table 4, p. 410)

bacteria, the sharing of minor somatic factors by certain species, or groups within the species, will reveal phylogenetic relationships. Conversely, removal of the group factors by preabsorption of antisera may yield useful reagents for species analysis (McCoy and McClung, 1938).

The repeated occurrence of the same flagellar (H) and somatic (O) antigens among the various species and types of Gram-negative bacilli can hardly be due to mere chance. In fact, studies of the antigenic structure, biochemical properties and pathogenicity of these organisms, reveals an almost complete intergradation of forms, leading to the view that the salmonella, the colon and paracolon bacilli, the shigella, in fact, the entire group

developed against each one of these capsular substances, however, fail to attack the other; in other words, they are even more specific than are the antibodies obtained by immunization of experimental animals. When treated with the specific polysaccharidases, virulent pneumococci lose simultaneously their capsules and, in a large measure, the property to agglutinate in specific antiserum; moreover, the enzymes confer upon animals a marked degree of protection against experimental infection with virulent pneumococci of the proper type (Avery and Dubos, 1931; Dubos and Avery, 1931; Dubos, 1932, 1935, 1939; Francis, Terrell, Dubos, and Avery, 1934; Goodner and Dubos, 1932; Goodner, Dubos, and Avery, 1932; Shaw, 1937; Sickles and Shaw, 1933, 1934, 1935, 1941).

The specificity of the reaction leaves no doubt that the destruction of the capsule revealed by staining techniques, the loss of precipitability of the capsular polysaccharide in specific antiserum, and the protection of animals against experimental pneumococcal infection, are all referable to the hydrolysis of the capsular polysaccharide. It must be emphasized that the enzymes are neither bacteriostatic, nor bactericidal; they destroy the capsules without affecting either the viability of pneumococci or their capacity to produce the capsular polysaccharide. From the point of view of cellular structure, therefore, it appears that the capsule is not a component essential to the integrity of the cell. It behaves as an excretion product which, because of its viscosity, accumulates around the cell wall and exerts an antiphagocytic effect which is one of the conditions of virulence (Chapter VI:2).

Effect of the Autolytic Enzymes upon the Antigenicity of Encapsulated Pneumococci.—Although the purified capsular polysaccharides react *in vitro* with the antibodies obtained by immunizing experimental animals with heat killed pneumococci of the homologous type, these same substances are not capable of eliciting the production of precipitating antibodies when injected into rabbits. Furthermore, autolysates of encapsulated pneumococci, which still contain the capsular polysaccharide, but in which the cell bodies are disintegrated, do not function as specific



FIG. 15.—Electron micrograph of *Eberthella typhosa* exposed to antityphoid serum. Flagella are thickened, opaque, fuzzy, although the picture is in very sharp focus (From Mudd and Anderson, 1941, fig. 4, p. 255.)

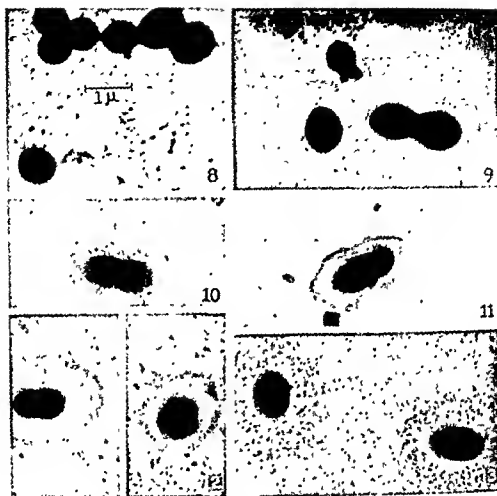


FIG 16—Electron micrograph of the pneumococcal capsular swelling reaction 8 *Pneumococcus* Type I Not exposed to serum. $\times 10,500$. 9 *Pneumococcus* Type I after 2 minutes' exposure to purified rabbit antibody globulin in 0.9 per cent NaCl solution containing 8.6 mg. antibody per 100 ml $\times 10,500$. 10 *Pneumococcus* Type I after 2 minutes' exposure to purified globulin solution containing 21.5 mg antibody per 100 ml $\times 10,500$. 11 *Pneumococcus* Type I after 2 minutes' exposure to purified globulin solution containing 43 mg. antibody per 100 ml $\times 10,500$. 12 *Pneumococcus* Type I after 3 minutes' exposure to 0.9 per cent NaCl solution containing 11 mg purified globulin per 100 ml and normal rabbit serum in final dilution of 1:4 $\times 10,500$. 13. *Pneumococcus* Type I after 3 minutes' exposure to 0.9 per cent NaCl solution containing 11 mg purified globulin per 100 ml. $\times 10,500$ The originals of these electron micrographs were obtained through the generosity of Dr. Stuart Mudd of Philadelphia, Pennsylvania (From Mudd, Heinmets, and Anderson, 1943, pl. 13, p. 332)

antigens in the rabbit (Avery, 1932, 1933; Avery and Neill, 1925). It appears, therefore, that in the intact pneumococcal cell, the capsular polysaccharide exists in an antigenic form different from that in which it has been isolated as a purified soluble substance. Indeed, it has been shown that pneumococci contain an enzyme which is capable of inactivating the capsular antigen, without destroying the capsular polysaccharide itself (Dubos, 1937d).

As already mentioned, the rapidity with which pneumococci undergo cytolysis is due to the presence in these bacteria of very active autolytic enzymes (Chapter IV:1). Virulent pneumococci treated with bile or bile salts undergo dissolution within a few minutes; flash lysis can also be observed when pneumococci desiccated with cold acetone are resuspended in neutral aqueous media and cellular disintegration is readily obtained by repeated freezing and thawing. All these procedures cause lysis through the action of one at least of the autolytic enzymes, and they all result in the inactivation of the capsular antigen although they leave the capsular polysaccharide itself in a form precipitable by specific antiserum. The inactivating effect of the autolytic system on the complete antigen can be demonstrated by a more direct technique. *Pneumococcus autolysates* are capable of causing the enzymic lysis of heat killed pneumococci, the lysis expressing itself by a loss of the Gram-positiveness, a disintegration of the cell body, and a clearing of the bacterial suspension. Moreover, when virulent pneumococci, killed by heat, iodine, or formal, are subjected to the action of active preparations of this bacteriolytic enzyme, they lose the property of eliciting in rabbits the formation of precipitating antibodies for the type specific polysaccharide. It is not necessary to cause a complete enzymic dissolution of the cell bodies in order to destroy antigenicity. It is sufficient to render the cells Gram-negative, without changing their characteristic morphology, as can be achieved by using small amounts of purified preparations of the autolytic enzyme (Dubos, 1937d; Meyer, Dubos and Smyth, 1937).

There exist other techniques which cause a limited autolysis

associated with complete loss of type specific antigenicity. Virulent pneumococci resuspended in a solution of formaldehyde or of iodine, or in a buffer at pH 4.0, remain Gram-positive and fully antigenic as long as they are maintained in these media. If the cells are now separated by centrifugation, and resuspended in a neutral buffer, they undergo an enzymic alteration which renders them Gram-negative and somewhat smaller. In each case, the enzyme which had been held in abeyance by formaldehyde or iodine, or by the acid reaction, recovers its activity in neutral solution. In each case, also, the change in staining reaction, although not resulting in dissolution of the cells, is accompanied by a loss of type specific antigenicity (Dubos, 1937d, 1938a).

Effect of a Heat Resistant Tissue Enzyme on the Specific Antigenicity of Pneumococci.—There exists in animal tissues an enzyme which decreases the basophilic character of heat killed pneumococci (as determined by affinity for basic dyes), and which inactivates at the same time their specific capsular antigen. This enzyme preparation, however, does not cause any alteration of the gross morphology of the cell, and does not decompose the capsular polysaccharide itself. The enzyme is extremely resistant to heat, especially at acid reactions, and the most purified preparations depolymerize ribonucleic acid, although they are completely inactive against other chemical substrates so far tested. These facts suggest that the cellular structure attacked by the enzyme contains ribonucleic acid, and it is known, indeed, that this substance is abundantly present in pneumococci. It must be realized, however, that even crystallized preparations of ribonuclease can be contaminated with other enzymes possessing similar physicochemical properties (lysozyme, for example) (Chapter IV:1). Only by testing the enzyme preparations upon a great variety of substrates, and by comparing the relative activity of different preparations against ribonucleic acid and against the specific antigen of pneumococcal cells, will it be possible to decide whether these two types of substrates are attacked by one and the same agent (Dubos, 1937a; Dubos and MacLeod, 1938; Dubos and Thompson, 1938).

Remarks on the Nature of the Capsular Antigen of Pneumococci.—Both the autolytic enzyme and the heat resistant tissue enzyme (ribonuclease?) mentioned above inactivate the capsular polysaccharide antigen without destroying the capsular polysaccharide itself. Moreover type III pneumococci (killed with heat, iodine or formaldehyde) treated with the polysaccharidase which hydrolyses the free type III capsular substance, retain their characteristic morphology and staining reactions, and in particular, their specific antigenicity in the rabbit. On the other hand, the killed cells of pneumococci are very susceptible to the action of trypsin. When a mixture of trypsin and chymotrypsin is added to a suspension of heat killed pneumococci, there occurs a rapid clearing of the cell suspension which leaves an insoluble residue containing only 22 per cent of the original cell weight with a total nitrogen content of 8 per cent. Microscopic observation reveals that the digested cell suspension consists of cocci of very small size, but which are still capable of retaining the Gram stain. Injection of the digested cell suspension into rabbits by the intravenous route gives rise to the production of type specific antibodies, indicating that the type specific antigen is extremely resistant to proteolytic action (Dubos, 1937c, d, 1939). These observations do not reveal the chemical nature of the complete capsular antigen of pneumococci. They establish clearly, however, that the free soluble capsular polysaccharide itself is not the complete antigen, and that the latter, although intimately associated with the cellular structure, is not a part of those proteins which are readily hydrolyzed by trypsin and chymotrypsin.

Unfortunately, the chemical nature of the substrate attacked by the autolytic system which destroys antigenicity is not yet known. One of the enzymes present in pneumococcus autolysate is capable of releasing reducing sugars from certain acetyl amino glucose glucuronides of animal tissues, but there is no indication that it is the same enzyme which also renders Gram-negative the heat killed pneumococci (Meyer, Dubos, and Smyth, 1937; Meyer, Smyth, and Dawson, 1938). It appears probable, however, that although the capsular polysaccharides vary in com-

position from one pneumococcal type to another, the capsular antigens themselves possess in their structure one constituent or linkage common to all pneumococci. This is shown by the following facts. All pneumococci (capsulated or not capsulated) contain an enzyme which is capable of inactivating the capsular antigens of all types. The antigen of all types can be inactivated by a heat resistant enzyme (ribonuclease?) extracted from animal tissues. All the agents or procedures which change pneumococci from the Gram-positive to the Gram-negative state cause at the same time inactivation of the capsular antigens. These agents and procedures are effective not only upon capsulated, but also upon the noncapsulated pneumococci. It appears, therefore, that the capsular antigens possess a complex structure, one part of which is a constant component of all pneumococcal cells, while another part, which is present only in encapsulated cells, varies in composition from one type to the other, and confers upon each individual type the immunological specificity characteristic of the homologous capsular polysaccharide.

4. SPECIFICITY OF BACTERIOPHAGE ACTION

Antigenic Structure and Specificity of Bacteriophage Action.—Pure lines of bacteriophage exhibit specificity with reference to the bacterial species which they affect; a staphylococcus bacteriophage is inactive, for example, against spore formers or typhoid bacilli. Indeed, this specificity is so narrow that it must be defined, not in terms of bacterial species, but rather in terms of strains and even of variant forms of one strain. Thus, a pure line of bacteriophage very active against a certain strain of *E. coli* can be inactive against another; or again, a bacteriophage which causes the rapid and complete lysis of the smooth variant of a certain bacterial culture can fail to affect, or to multiply on, the rough form of the same culture.

In contrast to these manifestations of specificity, it is possible to obtain bacteriophages which can attack several different bacterial strains provided these all possess a common antigenic con-

stituent. In the salmonella, for example, the presence of a given O or Vi antigen in otherwise different strains renders all of them susceptible to the bacteriophage specific for this cellular component. Thus, there exists a bacteriophage which can lyse not only *Eberthella typhosa*, but also *S. enteritidis* and *S. pullorum*, three bacterial cultures which possess the antigen IX of the Kauffmann-White classification. On the contrary, three other salmonella types not possessing antigen IX were found resistant to the above bacteriophage. The following observations provide additional evidence of the correlation between possession of a certain antigenic component and susceptibility to a certain strain of bacteriophage.

In the salmonella the change from the S to the R phase is associated with the loss of the specific somatic O antigens, and it is not surprising, therefore, that the R bacterial variants of susceptible S forms are resistant to those strains of bacteriophage, the specificity of which appears to be correlated with certain specific O antigens. It is known, furthermore, that the R variants of different salmonella types are characterized by a certain component, the R antigen, which is common to all types, and it has been observed that a bacteriophage capable of attacking one R variant can also attack the others. In other words, the specificity of the lytic agent can be correlated not only with the specific O antigens, but also with the R antigen (Almon and Stovall, 1939; Burnet, 1927, 1929a, 1930b, 1934; Rakieten and Rakieten, 1937a, b; Sertic and Boulgakov, 1935).

The intimate relationship between bacteriophage sensitivity and antigenic structure has also been confirmed by adsorption and inhibition experiments similar to those used in immunological analysis. In many cases, the lytic agents can be absorbed specifically by the cells, living or heat killed, of the susceptible bacterial culture, and this property has permitted an analysis of the bacteriophage receptors present in bacteria, analogous to the analysis of antigenic structure carried out by the adsorption of antibodies with bacteria. Thus, heat killed bacteria of the salmonella group absorb the different bacteriophages specifically in accordance with their antigenic structure as revealed by agglutination tests. As in

immunological reactions, results parallel to those of absorption tests can be obtained through the inhibition of lysis by different bacterial extracts. It has been found, for instance, that certain soluble fractions extracted from salmonella and shigella specifically inhibit the lysis of the homologous organisms by bacteriophage, and there is some evidence that the inhibitory substance is in some cases the polysaccharide of the specific antigen (Freeman, 1937; Gough and Burnet, 1934; Levine and Frisch, 1934, 1935; Levine, Frisch, and Cohen, 1934; Maitra, 1939; Rakieta and Tiffany, 1938).

Pure Lines of Bacteriophage as Specific Reagents.—The specific absorption of bacteriophages by the heat killed cells of susceptible cultures, and the specific inhibition of lysis by soluble extracts of susceptible bacteria, suggest that the specificities of bacteriophage and of immunological reactions depend upon the same cellular components. It appears, therefore, that the phenomena of phagolysis can be used as specific tests in the analysis of cellular structure.

It must be pointed out at this time that the parallelism between bacteriophage susceptibility and known antigenic structure is not always perfect. There has been described, for example, a resistant strain which appeared in a susceptible culture as the result of the lytic action of a particular bacteriophage and which failed to absorb the lytic agent, although it could not be differentiated by serological tests from the susceptible parent strain (Burnet, 1929a). Failure to correlate the development of resistance with modification of antigenic structure may be due in some cases to the fact that the receptor involved cannot be, or has not yet been, demonstrated by immunological methods. In the case of *S. cholerae suis*, it was only after the different strains of this species had been separated by their selective susceptibilities to different bacteriophages that the partial antigens VI₁ and VI₂ which differentiate them were recognized (Levine and Frisch, 1936). The fact that a certain staphylococcus bacteriophage can be absorbed by an unrelated culture of enterococcus has been explained by the presence in the latter culture of a substance, as yet unidentified but

probably of protein nature, which inactivates the lytic agent (Rakieta and Tiffany, 1938).

The division of the Vi strains of typhoid cultures into several substrains by their differential susceptibilities to pure bacteriophage lines presents problems of great theoretical and practical interest. Although no serological differences have yet been demonstrated between these cultures, it is possible that the cell receptors responsible for bacteriophage specificity could be identified by adequate absorption and inhibition techniques. That the Vi antigens themselves are not the receptors is suggested by the observation that some Vi strains become resistant to lysis without undergoing the Vi \rightarrow W variation which corresponds to loss of Vi antigen. Conversely, there has been discovered a bacteriophage which can differentiate between different Vi types, although it can also act on W forms (free of Vi antigen) of *E. typhosa* (Craigie and Yen, 1938; Dolman, Kerr, and Helmer, 1941).

The secondary bacterial growth which often appears in cultures that have undergone bacteriophage lysis consists of bacteria which are resistant to the strain of bacteriophage that caused the lysis, but which usually remain susceptible to other strains of bacteriophage, a fact which permits the isolation of a number of variants of one given culture characterized by differential susceptibilities (Bail, 1923). Thus, a culture of cholera vibrio subjected under the proper experimental conditions to three different bacteriophage lines, gave rise to four variants resistant to any one of the three agents or to all of them (Aschehov, *et al.*, 1933). In some cases, resistance may be due to the loss by the bacteria of the receptor to which the bacteriophage becomes bound or through which it gains entry into the cell. In other cases, resistance may be the result of the appearance of a new surface component which masks the specific receptor and thus causes steric hindrance of the lytic action. Under the influence of bacteriophages, for example, some salmonella strains, especially of *S. paratyphi* B, often yield a resistant growth which is of mucoid character, *i.e.*, which probably produces capsular material (Burnet, 1933).

The use of bacteriophage in the analysis of cellular structure

has much in common with the other biological methods previously considered. All these methods depend upon the reaction between a certain reagent (enzyme, antibody, or bacteriophage) and a specific cellular receptor, the reaction manifesting itself by enzymic destruction of a cellular component, by immune agglutination, precipitation, bacteriolysis, or by bacteriophage lysis. In certain cases, the reagent can be absorbed on the homologous cell substrate, or the reaction can be inhibited by the addition to the system of the specific substance which constitutes the cellular receptor. Enzymic decomposition, agglutination, precipitation, inhibition of growth, lysis, etc., are only the secondary manifestations of a primary reaction, the specificity of which depends upon the union between the cellular receptors on the one hand, and the biological reagent, be it enzyme, antibody, or bacteriophage, on the other.



THE VARIABILITY OF BACTERIA

Life's mystery is not truly manifested in adult forms, but according to my way of thinking, resides essentially in the reproductive cell and in its capacity for future development.

LOUIS PASTEUR (1875)

1. PLEOMORPHISM AND MONOMORPHISM

THE doctrine of variability of bacterial species found its most extreme expression during the early days of bacteriology in the school of pleomorphism. The different bacterial types were then assumed to be the multiple manifestations—morphological and biochemical—of only one or a very few species which could be changed the one into the other merely by changing the conditions of growth. It was soon established, however, that many of these early claims were based upon the result of faulty techniques, due to the experimental difficulties involved in obtaining and maintaining pure cultures. The criteria and methods introduced by Cohn (1872) and Koch (1876, 1881) left no doubt as to the existence of a multiplicity of bacterial types well defined in terms of the fixity of their morphological and biochemical characters (Bullock, 1938). The school of monomorphism thus introduced into bacteriology a much needed discipline which led to the separation and cultivation in pure cultures of the various agents associated with biochemical and pathological phenomena. It must be admitted, on the other hand, that the blind acceptance by several generations of bacteriologists of the Cohn-Koch dogma of constancy of cell forms and immutability of cultural characteristics discouraged for many years the study of the problems of morphology, inheritance, and variation in bacteria.

The isolation of a pure culture does not merely consist in the elimination of unrelated microbial species. It involves a process of domestication, an adaptation of the culture to life on a certain medium and under certain conditions (Parr and Robbins, 1942). Pure cultures of microorganisms, even issued from single cells, do not consist of identical individuals, and bacteria are more striking in their variability and plasticity than in the fixity of their morphological, biochemical and physiological characteristics. The pure culture technique results in the selection of a population made up to a large extent of the type of cells best adapted to a given environment, and any change in the latter is likely to be reflected in some modification of the appearance and of the very constitution of the cells. Moreover, the characters of a pure culture do not always remain unchanged even when the conditions of growth appear well defined and stable. Transformations—permanent or transient—not only of a quantitative, but often of a qualitative nature—appear in an unpredictable manner under conditions where the "purity" of the culture cannot be doubted.

Bacterial variability was noted and utilized from the very beginning of experimental bacteriology. In the morphological domain, Pasteur (1881) standardized techniques for the production of asporogenous forms of the anthrax bacillus. Colonial variation in vibrios was recognized as early as 1888 (Firtsch, 1888), and beautiful photographs illustrating variations in cellular and colonial morphology were published by Beijerinck long before rough and smooth colony variants received widespread recognition (Beijerinck, 1901, 1912). In the biochemical field, the specific modification of enzyme production by microorganisms as a response to the presence of a given substrate in the medium was discussed by Duclaux (1883, 1899) and by Wortman (1882) forty years before the expression "adaptive enzyme" was coined. Neisser (1906) and Massini (1907) described the appearance of lactose-fermenting variants in cultures of the nonlactose fermenter *E. coli mutabile*, and similar examples of other more or less permanent changes in the fermentative capabilities of bacteria soon flooded the bacteriological literature. It is hardly necessary to mention

that the discovery of avirulent forms of the etiological agents of fowl cholera, anthrax, swine erysipelas, etc., introduced very early into medical bacteriology the notion of instability of virulence as a factor to be considered in epidemiological analysis, and as a technique in the practice of immunization (Konst, 1940).

It is obvious, therefore, that the doctrine of monomorphism must be restated in a form which will make it possible to encompass in the definition of any one culture the different genotypes and phenotypes which that culture is potentially capable of producing. Students of the problems of bacterial variability have borrowed from the science of genetics a number of words such as mutations, genotypes, phenotypes, etc. Although this practice is often necessary for reasons of convenience, it must be realized that the terminology of classical genetics may not be entirely applicable to bacteria, since it has not yet been convincingly proven that these organisms reproduce by sexual mechanisms, and since so little is known of the structure and behavior of their nuclear apparatus (Chapter II:2). In any case, the manifestations of bacterial variability are, outwardly at least, very similar to those recognized in other organisms. They include the transient modifications which are a direct effect of the environment, and the more or less permanent variations which often occur spontaneously in an unpredictable manner. Before attempting to define the range and mechanism of bacterial variation, however, it is important to point out that bacteria also exhibit orderly, predictable changes which are related to the age of the culture and which are the expression of a definite growth cycle.

2 VARIATIONS OF THE BACTERIAL CELL DURING THE GROWTH CYCLE—CYTOMORPHOSIS

He who watches a thing grow has the best view of it.

HERBERT OF EMBURY

Experimental studies of the morphology and physiology of unicellular organisms have provided convincing evidence that the cells of early generations developing in a fresh, favorable

medium are quite different from those of generations developing after a period of maximum multiplication. Whether the growth process is followed by stained preparations, projected images or photomicrographs of organisms treated with Congo red, con-

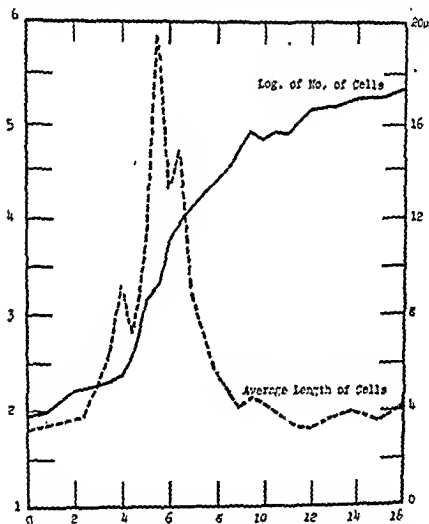


FIG. 17.—Growth curve and variations in size of cells of *B. megatherium*: culture I. (From Henrici, 1928, fig. 14, p. 70)

secutive records obtained by means of motion photomicrography, measurement of opacity of cultures, or some other means, all bacterial species exhibit the same behavior, with the possible exception of the diphtheria bacillus. The mean cell volume increases during the lag period of culture development, and reaches

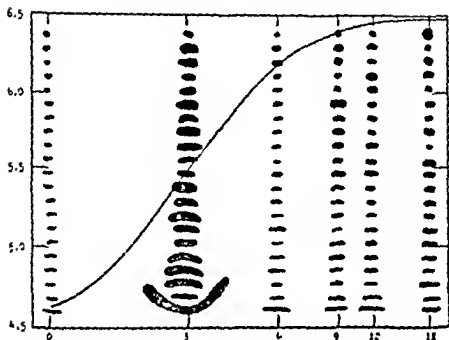


FIG. 18.—Representative cells from a culture of *B. coli* at different stages of growth (From Henrici, 1923, fig. 25, p. 89)

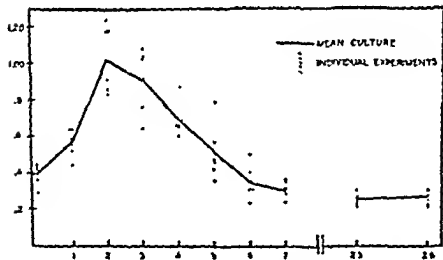


FIG. 19.—Cell volume of *E. coli* in peptone water by hours (From Huntington and Winslow, 1937, fig. 1, p. 130)

a maximum before multiplication occurs at the maximal rate; it diminishes during the logarithmic phase of rapid multiplication, and reaches a minimum when the culture stops growing. The ratio between minimal and maximal cell size varies from 1:3 to 1:5 according to the species (Adolph, 1931; Alper and Sterne, 1933; Bayne-Jones and Adolph, 1932; Hershey and Bronfenbrenner, 1938; Henrici, 1928; Huntington, and Winslow, 1937; Jensen, 1928; Regnier, Lambin, and Jund, 1938; Winslow and Walker, 1939). (Table 16.)

TABLE 16

SIZE OF CELLS (MICRA) OF THREE DIFFERENT CULTURES OF *B. Megatherium*, OF DIFFERENT AGES

HOURS	'STRAIN 1	STRAIN 2	STRAIN 3
0	3.4	5.2	5.2
5	11.3	7.6	9.6
10	4.5	5.0	5.9
20	3.8	3.6	4.4

Data from Winslow and Walker (1939, Table 5, p. 179).

Young and old cells differ in many respects other than size. Young cells exhibit greater affinity for the basic dyes and are less readily agglutinated by acid, suggesting an iso-electric point of the protoplasm more on the acid side, a property probably correlated with the greater electropositive character revealed by the rate of migration in an electric field (Choucrroun, 1938; Moyer, 1936; Regnier, Lambin, and Jund, 1938; Seigneurin, 1938). Oxygen uptake, production of heat, carbon dioxide, and ammonia, indicate that young cells metabolize more rapidly, although it is still uncertain whether the increased metabolism during the lag period and early part of the logarithmic growth phase is due only to increased cell volume or to a physiologically different state (Clifton, 1937; Hershey and Bronfenbrenner, 1938; Huntington and Winslow, 1937). (Table 17.)

TABLE 17

REPRODUCTIVE RATE, CELL VOLUME AND METABOLIC ACTIVITY OF *E. coli*
(Maximum value in heavy type)

Time*	PEPTONE			PEPTONE GLUCOSE		
	Metabolic Activity†	Cell Volume‡	Reproductive Rate§	Metabolic Activity†	Cell Volume‡	Reproductive Rate§
0		.39			.41	
1	116	.57	.02	78	.77	-.13
2	135	1.04	.40	88	1.03	1.04
3	90	.91	.92	79	.91	1.41
4	100	.69	1.17	59	.91	.64
5	95	.52	.56	31	.81	.42
6	68	.36	.94	21	.84	.04
7	43	.31	.45	19	.59	.15
24		.26			.76	
25	8	.27	-.60	7	.75	.05

* Interval after inoculation in hours

† Milligrams $\times 10^{-11}$ of CO_2 per cubic micron per hour for preceding hour.

‡ Mean cell volume in cubic micra at stated hour

§ Reproductive rate during preceding hour. Computed from formula:

$$\frac{1}{t} \times \ln \frac{b}{B}$$

where B and b equal initial and final numbers.

Data from Huntington and Winslow (1937, Table 2, p. 131).

The cells are most adaptable to new types of food during the lag phase, before the maximal growth rate or the logarithmic phase is reached. Adaptability decreases rapidly during the logarithmic phase, while the rate of growth remains constant. These changes are probably correlated with the greater ability for the adaptive production of enzymes exhibited by cultures just coming out of the lag phase (Hegarty, 1939). At the end of the lag period and during the logarithmic growth phase, on the other hand, the cells are less resistant to a great variety of inimical agents and procedures—phenol, crystal violet, salt concentrations, heat, cold, etc.—than are those of older populations (Illiker and Frazier, 1938; Hegarty and Weeks, 1940; Hentrich, 1928; Sherman and Brooks Naylor, 1942; Winslow and Walker, 1939). (Table 18.)

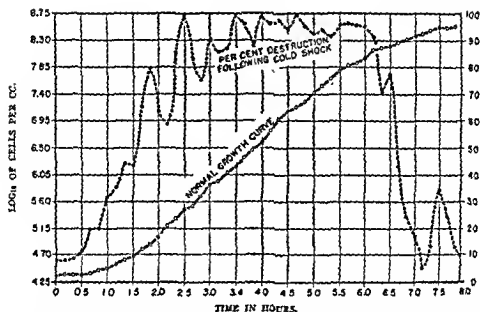


FIG. 20.—Per cent destruction effected by cold shock at ten minute intervals during the normal growth cycle. (From Hegarty and Weeks, 1940, fig. 1, p. 477.)

TABLE 18

HEAT RESISTANCE OF CULTURES OF *Escherichia coli* GROWN AT 28° C. FOR VARYING PERIODS AND THEN HEAT SHOCKED AT 53° C. FOR THIRTY MINUTES

AGE OF CULTURE	PLATE COUNT		PERCENTAGE SURVIVAL
	Before Heating	After Heating	
Hours	Nos. per cc.	Nos. per cc.	
0	25,200	1,600	6.4
1.5	33,800	11,500	34.0
3	40,350	550	1.4
6	297,000	320	0.11
9	730,000	140	0.02
12	1,350,000	540	0.04
15	2,730,000	5,120	0.19
18	3,410,000	58,000	1.7
21	3,000,000	55,000	1.8
24	3,170,000	71,800	2.3
27	3,240,000	83,000	2.6
30	2,710,000	129,000	4.8
33	2,560,000	184,000	7.2
36	3,200,000	184,000	5.8

Data from Elliker and Frazier (1938, Table 3, p. 89).

Evidence that the bacterial cell undergoes with age an orderly modification of its morphological and physiological properties has been obtained by other techniques. It has been claimed, for example, that if the distal elements are constantly removed from a culture of *B. subtilis* so as to retain only the younger cells, or, on the contrary, if the medial elements are removed to keep only the older extremities of the growth, the distal elements stop dividing at a time when the younger medial elements are still multiplying. Death is observed much more frequently (thirty times more often) in the distal than in the medial elements (Koblmüller, 1937). Similar observations have also been made with yeast. It can be shown by isolation of single cells that the older forms exhibit a slower multiplication rate and that this slower rate is transmitted to new yeast cells issued from them (Schoutens, 1935).

There are thus a number of properties which are correlated with the early growth period: increased length and slenderness of the cell, indicating a greater magnitude of some axially disposed force opposing the surface tension of the medium; increased intensity of staining with basic dyes and change in electrokinetic mobility indicating an iso-electric point of the protoplasm more on the acid side; increased metabolic activity; increased susceptibility to injurious agents and procedures. It is to account for these profound changes in morphology and behavior that some authors have postulated that bacterial cultures exhibit a period of physiological youth, resembling that of more highly differentiated multicellular organisms, and during which bacteria can be considered as being in an embryonic phase (Henrici, 1928; Sherman and Albus, 1924; Winslow and Walker, 1939).

1. NONTRANSMISSIBLE MODIFICATIONS INDUCED BY THE ENVIRONMENT

Many bacterial species, even among the pathogens, are capable of multiplying under an extremely wide range of conditions—temperature, salt concentration, acidity, nature of the nutrients, etc. In many cases, growth in a new environment results—either by

some direct effect on the genetic constitution, or more likely by the selection of some naturally occurring mutant—in the manifestation of new characters which the cell is capable of transmitting to its progeny. In other cases the modifications induced by the environment are extremely transient, and the cell reverts to its original form as soon as it is allowed to grow again under the original conditions. A few illustrations of these modifications which are immediately and completely reversible will be given in the following pages.

Effect of the Environment on Some Physicochemical Characters of Bacteria.—The elongated form of bacillary species is due to an internal axially disposed force which opposes the rounding effect of surface tension. It is not surprising, therefore, that when bacteria are placed in a medium of lowered surface tension, this force meeting less opposition produces a more pronounced effect, giving rise to cells which are even longer and more slender. Thus, young cells of *E. coli* growing on sodium ricinoleate agar appear as long and even filamentous forms which extend over several microscopic fields. On the contrary, when calcium chloride, which raises somewhat the surface tension, is added to the medium, the cells are shorter and more oval than in the normal medium (Frobisher, 1926; Hentici, 1928). It may be also because of their ability to depress surface tension that digitonin and especially convallamarin—added to glycerol-potato media—cause the tubercle bacillus to grow in a non acid-fast form, which can readily be suspended in homogeneous emulsions (Hasegawa and Kochi, 1939).

Although most bacteria can adapt themselves to an extremely wide range of osmotic pressure, the cellular morphology of organisms of the hemorrhagic septicemia group is affected by large concentrations of electrolytes which cause the appearance of forms abnormal by their large size and pleomorphism. The concentration of electrolytes also influences capsule formation by *Klebsiella pneumoniae* (Hoogerheide, 1939, 1940). Certain inorganic elements exert a specific effect on the characters and properties of bacterial cultures. For example, deficiency of the culture

medium in calcium results in increased sporulation and mucoid growth of the anthrax bacillus (Bordet and Renaux, 1930); inorganic iron exerts a dramatic effect on toxin production by certain pathogens, etc. (Chapter VI:4).

The effect of the gaseous environment could also be illustrated by many examples. Reduced oxygen tension decreases the pigmentation and increases the smooth character of colonies of the tubercle bacillus (Vera and Rettger, 1940), sporulation of the anthrax bacillus is inhibited by high CO_2 pressures which, on the other hand, stimulate capsule production (Ivánovics, 1937; Sterne, 1937, 1938); toxin production by the staphylococcus and the Shiga bacillus is conditioned by oxygen and carbon dioxide tension (Chapter VI:4).

Organic substances obviously affect many of the characters of bacterial growth, ranging all the way from the production of pleomorphism in cultures of cholera vibrios by glycine and alanine (Gordon and Gordon, 1943) to the production of serologically active polysaccharides by streptococci of group II in the presence of sucrose but not of dextrose (Neill, Sugg, Hehre, and Jaffe, 1941). Temperatures and different radiations can also affect morphology, pigmentation and other properties of bacteria, and it has even been claimed that the cellular dimensions and metabolic activities of a number of bacterial species are under the influence of the solar spot cycles (Tehijevsky, 1938).

Analysis of the mechanism of adaptation of *E. coli* to increasing concentrations of sodium chloride has revealed that mere exposure of nondividing cells to gradually increasing concentrations of salt, or even to a single intermediate NaCl concentration, is sufficient to bring about an acclimatization which occurs without cell reproduction. The rate of the individual acclimatization process increases with temperature; it occurs more readily in cultures in the early stationary phase and becomes less efficient during the logarithmic phase and senescence. Acclimatized bacteria rapidly lose their increased ability to reproduce in saline media upon return to a salt-free environment, even when no reproduction of the cells can be detected (Doudoroff, 1940).

TABLE 19

REVERSIBILITY OF ACCLIMATIZATION OF *E. coli* TO NaCl

		TIME	BACTERIA VIABLE IN 6.5 PER CENT NaCl BROTH	
		hrs.		
Before acclimatization			9.5×10^8	
After acclimatization		0	2.5×10^8	
			Bacteria resuspended in buffer	Bacteria resuspended in 1.5 per cent NaCl
"	"	1	2.5×10^8	7.5×10^7
"	"	3	4.5×10^8	2.5×10^8
"	"	6	2.0×10^8	9.5×10^7
"	"	20	4.5×10^8	2.5×10^8

Data from Doudoroff (1940, Table VII, p. 599).

It appears, therefore, that not only are some of the modifications directly induced by the environment not transmitted by the cell to its progeny, but that some of the processes involved are so readily reversible that return to the normal state occurs during the life of the individual cell.

Effect of the Environment on Enzyme Production.—The enzymic constitution of the bacterial cell can undergo a wide range of variation in response to alterations in the environment. In some cases, environmental factors determine a transmissible change in the organism; this subject will be considered later under the heading of "discontinuous variations" (Chapter V:4). Variation in enzyme production can also take the form of a specific and reversible response to the presence of a given substrate in the culture medium, giving rise to the adaptive production of enzymes which are selectively active against the substance with reference to which variation has taken place. In a more general way, however, any modification of the environment can bring about quantitative and qualitative alterations of the enzymic activity of the bacterial cell.

Inorganic elements like calcium, magnesium, or iron affect the production of gelatinase by proteus bacilli, of phosphatase by propionic acid bacteria, or of the lecithinase (α toxin) of the

Welch bacillus. Organic radicals can act as essential building stones for the synthesis of certain enzymes or modify their activity for other more or less defined reasons; essential growth factors (vitamin-like), in particular, are required to provide the co-enzymes of many cellular catalysts. Oxygen tension, acid base conditions, temperature of incubation, etc., all affect the production, stability and activity of the bacterial enzymes. Together with the other cellular and environmental factors, they determine what part of the metabolic potentialities of the species become actually expressed in a given culture (Dubos, 1940; Gale, 1943; Karström, 1937-38; Quastel, 1937; Yudkin, 1938).

There is, of course, "a limit to the total range of enzymes that any one organism can produce, and one organism may react to a given change in conditions in a way specifically different from another. Systematic bacteriology makes use of such differences for the separation of genera, species, and strains. Thus every organism has a repertoire of activities which it can produce, but the particular activities selected from the repertoire for inclusion in any one culture of that organism are determined by the conditions holding during the growth of that culture" (Gale, 1943).

Adaptive and Constitutive Enzymes.—There are certain enzymes which have been called "adaptive" because they appear as a specific response to the presence of the homologous substrate in the culture medium, and are thus differentiated from the "constitutive" enzymes which are always formed by the cells of a given strain, irrespective of the composition of the medium (Karström, 1937-38). The stimulating effect of a given substrate upon the production of the homologous enzyme can exert itself through a number of different mechanisms. We may be dealing, for example, with a culture capable of producing a variant form endowed with the proper enzymic property. If the homologous substrate is present in the medium, the variant will be favored to multiply rapidly, and the new enzyme will accumulate in the culture. Once so developed, this new enzymic function, which is the result of natural selection, usually remains stable for a number of generations even though the culture is from then on trans-

ferred into media not containing the substrate with reference to which variation has taken place.

The "adaptive" enzymes to be considered in the following pages exhibit a different behavior. They appear without delay when the cells of the proper microbial species start multiplying in a medium containing the specific substrate; the specific enzymic activities reach their maximum development during growth of the very first transfer into the specific media, and the enzymes again fail to accumulate as soon as the cultures are transferred to media lacking the specific substrates. (Table 20.)

TABLE 20

THE PRODUCTION OF LACTIC ACID BY *Streptococcus lactis* GROWN IN GLUCOSE OR GALACTOSE

PAST HISTORY OF CULTURE		MG. LACTIC ACID PRODUCED PER 100 CC. OF BACTERIAL SUSPENSION			
		1 hr.	2 hrs.	3 hrs.	4 hrs.
Cells grown with glucose, suspended in	glucose	240	360	420	470
	galactose	0	0	0	0
Cells grown in galactose, suspended in	glucose	160	300	370	420
	galactose	180	270	400	410

Data from Rahn (1938, Table on p. 363).

These phenomena are strikingly illustrated in the case of an organism capable of oxidizing p-aminobenzoic acid. (Table 21.)

Studies of the rate of development of the oxidizing system responsible for this action reveal that the nonadapted bacteria develop the specific enzymic activity after one hour's shaking with PABA. On the other hand, when the cells are removed from the reaction system after different intervals of time, the specific enzymic activity begins to decrease as soon as the PABA has been completely oxidized (Mirick, 1943) (figs. 21 and 22). Adaptive enzymes therefore appear to depend for their production upon the presence of the homologous substrate in the medium; they do not conform to the behavior of hereditary variations.

TABLE 21

SPECIFICITY OF STIMULATION OF THE PRODUCTION OF BACTERIAL ENZYMES
CAPABLE OF OXIDIZING PARA-AMINO BENZOIC ACID

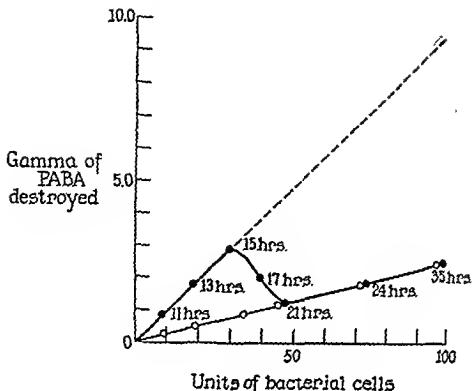
TYPE OF SUBSTANCE	SUBSTANCES WHICH STIMULATE PABA-OXIDIZING ENZYMES			SUBSTANCES WHICH DO NOT STIMULATE PABA-OXIDIZING ENZYMES		
	Substance	Activity ratio*	Amount [†] destroyed	Substance	Activity ratio	Amount destroyed
ISOMER	p-Aminobenzoic acid	25.0	50.0	m-Aminobenzoic acid	1.0	0
				Anthranilic acid	1.0	1.5
AMINO GROUP COVERED	Acetylated PABA	25.0	50.0	Benzoylated PABA	1.0	0
	Glycyl PABA	1.2	1.0			
AMINO GROUP OXIDIZED OR ABSENT	p-Nitrobenzoic acid	25.0	25.0	Benzoic acid	1.0	2.0
	p-Toluic acid	2.6	4	p-Hydroxybenzoic acid	0.7	2.0
ESTER	Methyl ester of PABA	2.0	1.0	Ethyl ester of PABA	1.0	0
	Novocaine‡	1.0	1.0			
COOH GROUP ABSENT OR MODIFIED	p-Aminophenyl acetic acid	1.3	1.0	Aniline	1.0	0
				p-Aminohyppuric acid	1.3	0
				p-Aminobenzylic alcohol	1.0	0
				p-Aminophenylalanine	1.0	0
				p-Aminophenylglycine	—	0
				p-Aminophenol	0.5	0
				Arsenic acid	0.4	0
				Sulfanilic acid	0.4	0
NO AMINO GROUP				Sulfanilamide	—	0
				Sulfapyridine	0.6	0
				Sulfathiazole	0.5	0
				Sulfadiazine	0.5	0

* Activity ratio = gamma of PABA oxidized in 30 minutes at 37°C. by 20 units of cells grown for 12 hours in casein hydrolysate medium containing 1 mg. per cent of the indicated substance, gamma of PABA oxidized under the same conditions by 20 units of cells grown in plain casein hydrolysate medium.

† Amount destroyed = gamma of indicated substance destroyed in 30 minutes at 37°C. by 20 units of cells specifically adapted to oxidize PABA.

‡ Activity possibly due to slow hydrolysis with the formation of free PABA.

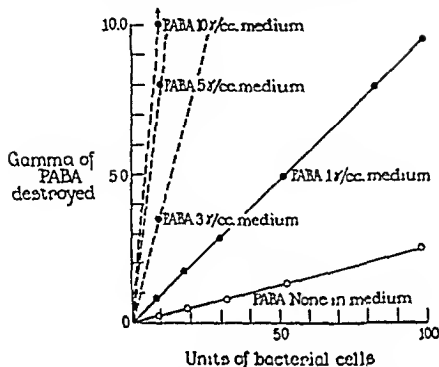
Data from Munk (1941, Table 1, p. 264).



- Cells tested after growth in a medium containing no PABA.
- Cells tested after growth in a medium originally containing 18/cc. of PABA all of which was destroyed after 11 hours growth.

FIG. 21.—Activity of soil bacillus in destruction of PABA. (From Mirick, 1943, fig. 1, p. 259.)

Mechanism of Production of Adaptive Enzymes.—It appears unlikely that a character acquired and lost so suddenly can be due to the natural selection of variant forms endowed with the new enzymic property. Against the natural selection hypothesis is also the fact that, in many cases, the newly acquired enzyme can hardly be of any value to the organism which produces it. Thus, *Escherichia coli* produces hydrogenlyase only when grown in a formate medium. The reaction $\text{HCOOH} = \text{H}_2 + \text{CO}_2$ which is catalyzed by this enzyme can at best liberate only very small amounts of free energy, and since it is unlikely that the products



- Cells tested after growth for 12 hours in medium containing no PABA.
- Cells tested after growth for 12 hours in medium containing varying amounts of PABA.

FIG. 22.—Effect of age of culture and presence of PABA in the growth medium upon PABA-oxidizing activity of soil bacillus. (From Mirick, 1943, fig. 2, p. 261.)

of the reaction are required by the organism for its growth, there is no obvious reason why cells endowed with the ability to produce hydrogenlyase should be selectively favored in the growth of a normal population. On the basis of the natural selection hypothesis, moreover, the immediate loss of hydrogenlyase when the organism is transferred to a medium deficient in formic acid would suggest that the loss of the enzyme is an advantage to the cells grown in plain broth, an assumption for which there is no ground. It appears unlikely, therefore, that the production of hydrogenlyase is due to natural selection (Yudkin, 1932, 1937c,

1938). The conversion of creatine into creatinine by an adaptive enzyme is another example of a reaction which appears to be of little use to the organism involved, and even more striking is the fact that this enzyme is formed as readily when creatinine (the end product of the reaction) instead of creatine is added to the medium (Dubos and Miller, 1938).

It is obvious that the natural selection hypothesis would be ruled out if the production of adaptive enzymes could be obtained in the absence of any cellular division. This result appears to have been achieved in the case of hydrogenlyase and of yeast galactozymase, on the following evidence: a) when washed cells not possessing the enzyme are resuspended in a solution of the specific substrate, enzyme formation can be demonstrated within one hour, *i.e.*, in a time too short for appreciable cell division to take place; b) it appears from viable and total cell counts during the adaptation, that enzyme production occurs without increase in cell numbers (Stephenson, 1937; Stephenson and Stickland, 1933; Stephenson and Yudkin, 1936).

These observations suggest strongly that enzyme production can occur in the absence of cell multiplication; they do not establish, however, that enzyme production does occur without the synthesis of new protoplasm. We have already mentioned that most bacterial cells, transferred to a new medium, undergo a phase of enlargement and elongation prior to cell division (Chapter V: 1). The metabolism of each individual cell increases during this period, a phenomenon probably associated with the production of new protoplasm not accompanied by cell division. It is certain, therefore, that production of new cell material occurs during the one-hour period required before production of new enzyme is observed. It must be mentioned in this respect that, in spite of many attempts, no production of adaptive enzymes has been observed in the presence of protoplasmic poisons or under conditions incompatible with cell growth (Dubos, 1935; v. Euler and Jansson, 1927; Stephenson and Yudkin, 1936). Even in the case of formic hydrogenlyase, no enzyme was formed unless some bouillon was added to the formate solution (Stephenson and Stickland, 1932, 1933). It seems, therefore, that although the adaptive pro-

duction of enzymes can occur in the absence of cellular division, it always involves the synthesis of new protoplasm.

Some authors have tried to describe the mechanism of enzyme production in chemical terms. Thus, enzymes can be considered as metabolites, whose rate of formation and destruction varies with the conditions of growth. The substrate could exert its action either by contributing the necessary organic molecules for the synthesis of the enzyme, or by affecting its stability (for instance, by combining with it). The adaptive stimulation caused by the homologous substrate would only be one particular application of these principles (Quastel, 1937). In fact, kinetic studies of the synthesis and destruction of the galactose fermenting system in a genetically stable yeast population suggest that one is dealing in this case with an enzyme system extremely unstable in the absence of its specific substrate, and that the difference between adaptive and constitutive enzyme may therefore be one of stability (Spiegelman, Lindegren, and Hedgecock, 1944). Support for this view may be found in the fact, already mentioned, that bacterial cells adapted to the oxidation of *p*-aminobenzoic acid begin to show a loss of the specific enzymic activity shortly after the substrate has disappeared from the medium as a result of complete oxidation (Mirick, 1943) (fig. 22).

A mass action theory of enzyme production has been formulated on the assumption that, in all cases, the stimulation caused by the proper substrate does not result in the production of a new enzyme, but only increases the production of an enzyme otherwise formed in small amounts. If the enzyme is in equilibrium within the cell with an inactive precursor, any substance combining with the active form will disturb the equilibrium and thus cause the production of more enzyme from the precursor; the adaptive stimulation by the homologous substrate could be explained on this ground (Yudkio, 1938). Although there are many facts in support of this theory, there are others which suggest that the formation of specific enzyme depends, not upon the mere presence of the homologous substrate in the medium, but upon its utilization by the metabolizing cell (Dubos, 1940).

It has also been suggested that the specific stimulation of en-

lactose agar, *E. coli mutabile* produces colonies which at first show no evidence of lactose fermentation. After some days, however, secondary papillae which are endowed with the ability to attack the dissaccharide grow out of the original, primary colonies. Subcultures of these secondary colonies on agar or in lactose broth give prompt and typical lactose fermentation and this character is faithfully reproduced in subsequent cultures. Because it is possible to train *E. coli mutabile* to acquire and to transmit to its progeny the ability to ferment lactose, merely by growing it in the presence of the sugar, it has often been concluded that the sugar exerts some sort of a specific, inciting stimulus to variation. It has also been suggested, on the other hand, that variation occurs spontaneously in the absence of the specific sugar, and that the latter acts only as a selective agent which favors the growth of the lactose positive variants. In order to establish whether cultures grown on plain agar really contain any cell capable of utilizing lactose, advantage was taken of the fact that some strains of *E. coli mutabile* can grow in synthetic media containing ammonia as sole source of nitrogen and a sugar as a sole source of carbon. On account of their importance, these experiments will be quoted directly from the original paper (Lewis, 1934).¹

"A 24-hour agar slant culture was washed down with 10 cc. of water and further diluted by steps of ten to include the complete range from one tenth to one billionth part of the culture. All dilutions were plated in glucose as well as lactose synthetic agar in order to determine with certainty that failure to obtain growth in the preceding experiments could not have been due to the nitrogen source. The highest dilutions were plated in peptone beef-extract agar to determine the number of cells introduced into the plates of lower dilutions. Computing from the plain agar series, it was estimated that the lowest dilution contained about three billion cells per cubic centimeter. Growth occurred throughout the range in glucose synthetic agar. The colonies in plain agar and glucose synthetic agar were practically equal in the high dilutions. Lactose synthetic agar supported growth in low dilutions only. Plates above the 100,000 dilution were sterile. The number



Fig. 24—Production of daughter colonies
by the mother of Dr. Roy Avery of

University of California



of colonies in plates which supported growth was proportional to the dilutions. It was determined that not more than one cell per 100,000 could grow in this medium.

"The colonies grew vigorously with formation of acid. Transplants to eosin-methylene-blue agar gave typical metallic colonies in some instances, while in others the colonies were purple or with purple centers. Shake cultures in lactose peptone agar produced acid and gas within 12 hours. It was thus shown that cells of two types were present in the original cultures. All cells capable of growth yielded races which could not be distinguished from variants isolated by plating from secondary colonies.

"There seemed no reason to believe that the culture consisted of a mixture of two unrelated strains. However this possibility had to be excluded before we could conclude that variation occurs spontaneously without the influence of lactose.

"Two methods of experimentation were available, namely, isolation of pure strains by single-cell methods or testing numerous colonies from ordinary plain agar plates. Since the latter appeared likely to afford reliable results, the experiments were carried out by this method. The previous experiment had shown that variants are present in small numbers only. It seemed, therefore, that plating to plain agar from high dilutions would insure separation of the two types. Accordingly, plate cultures were prepared from a 24-hour broth culture diluted one to one hundred million. Such plates contained about 8 to 10 colonies, not more than 2 or 3 of which grew on the surface. Each colony to be tested for variants was scraped off as completely as possible and emulsified in 100 cc. of sterile water. The number of cells capable of growth in lactose synthetic agar was determined by plating 1 cc. of the suspension in this medium and the total number by plating appropriate higher dilutions in beef extract peptone agar and glucose synthetic agar. The results are shown in Table 3." [Table 3 corresponds to Table 23 in the present text.]

"It is seen that all colonies contained variants and that the proportion of original to variant cells was relatively uniform.

"In addition to this experiment, numerous colonies from plain

agar plates were tested by taking up as much of the growth as was possible, suspending it in 10 cc. of water, and plating 1 cc. in lactose synthetic agar. This series included 100 colonies from plain agar plates prepared from high dilutions as described above. All colonies contained variant cells in about the same number per colony shown in Table 3. Thus it is proved that variation occurs in this strain in the absence of lactose. The variant cells, however, are so few in number as to escape detection by conventional methods of plating. (Table 23.)

TABLE 23

NUMBER OF VARIANT AND ORIGINAL TYPE CELLS IN PLAIN AGAR COLONIES OF *B. coli mutabile* AS DETERMINED BY CAPACITY FOR GROWTH IN LACTOSE SYNTHETIC, GLUCOSE SYNTHETIC AND BEEF EXTRACT PEPTONE AGAR

COLONY	KIND OF MEDIUM AND NUMBER OF CELLS PER COLONY CAPABLE OF GROWTH		
	Lactose synthetic agar	Beef extract agar	Glucose synthetic agar
1	6,300	3,540,000,000	3,420,000,000
2	3,300	1,610,000,000	1,640,000,000
3	2,900	730,000,000	710,000,000
4	2,200	840,000,000	860,000,000
5	2,080	1,200,000,000	1,100,000,000

Data from Lewis (1934, Table 3, p. 627).

"In order to determine whether variation could occur in a medium without either peptone or lactose, a culture was carried in glucose synthetic liquid medium with daily transplants for a period of ten days. At the end of this period the last tube of the series was plated in glucose synthetic agar. After an incubation period of 48 hours, single colonies were suspended in sterile water and plated in lactose synthetic agar. The number of variant cells was approximately the same as in colonies from beef-extract peptone agar. It appears that variation occurs under any conditions which are suitable for growth."

This variation apparently occurs without regard to environmental influences. Variation, when beneficial, may be preserved

by selective action of the medium. Nonbeneficial variation, such as poor adaptation to nutrients, would in general be eliminated by overgrowth of the more vigorous original cells (Lewis, 1934).

Other Examples of Discontinuous Variations in Enzyme Production.—The production of lactose positive variants by *E. coli mutabilis* is not a unique case; in fact, the same organism also varies with reference to several other saccharides, and to citrate. *S. paratyphi B* varies with reference to raffinose, and *E. typhosa* with reference to lactose, sucrose, rhamnose, dulcitol, and isodulcitol. In several of these cases also, variant cells occur in the parent culture (in plain broth or plain agar in the absence of the sugar), but in such small numbers as to escape detection by conventional methods (Lewis, 1934; Parr and Simpson, 1940; Penfold, 1911; Sage and Spaulding, 1942). *Salmonella dublin* can vary with reference to l-arabinose and here again the variation occurs in the absence of the pentose (Kristensen, 1940). A strain of *Shigella paradysenteriae* Sonne capable of variation with reference to four sugars: lactose, sucrose, raffinose, and maltose, gave several different variant strains, each possessing different combinations of the enzymes concerned (Sears and Schoolnik, 1936; Welch and Mickle, 1932).

A number of different organisms were found to be unable to initiate development in a synthetic medium containing d-arabinose as the only source of energy. Cultivation in the presence of this sugar gave variant forms capable of fermenting it rapidly, but which remained inactive toward the l-form. When filtrates of d-arabinose broth cultures from which the cells had been removed just prior to active fermentation were re-inoculated with new cells which had not previously been in contact with d-arabinose, fermentation was delayed. It appears, therefore, that the change consists in an alteration of the cells and not in a conversion of the d-sugar to some more readily available form before it is finally broken down (Koser and Vaughan, 1937).

Variation does not occur only with reference to sugars. Strains of clostridia can be adapted to more rapid utilization of gelatin, or casein (Kocholaty and Weil, 1938). *B. mycoides* can give sec-

ondary colonies on agar containing protein alone, variations apparently being related to some unused fraction of the split protein (Lewis, 1933). Bacteria can also exhibit discontinuous variation in their ability to synthesize complex organic compounds from simpler ones. Ordinary strains of *S. paratyphi A* and of *E. typhosa*, for example, can be transformed into strains capable of assimilating ammonia by heavy inoculation and serial transplants in media containing ammonia as the sole source of nitrogen. Neither organism is at first capable of growth in the medium when inoculated in the usual way, but when enormous numbers are introduced, a few cells are able to grow. Pure cultures of ammonia-assimilating strains can then be recovered (Braun and Cabn Bronner, 1922). The training of many organisms to become independent for their multiplication of some growth factor required by the parent strain—tryptophane in the case of *E. typhosa*, for instance (Fildes, Gladstone, and Knight, 1933)—is now explained by the ability of the variant strain to synthesize the growth factor in question (Knight, 1936; Leonian and Lilly, 1942). According to this view, many cultures contain a few cells endowed with special synthesizing powers, and selective multiplication of these cells permits adaptation to deficient media. It is very likely that methods similar to the ones used for the study of the mechanism of variation to lactose exhibited by *E. coli mutabile* would also reveal the existence in the parent culture of a few mutants endowed with the specific synthetic powers in question.

Progressive Variation.—There are many examples of training which cannot be readily defined in terms of an enzyme concerned in some specific metabolic action. Such are, for instance, the increase in resistance, often of lasting character, which follows exposure of a culture to some toxic substance or injurious procedure, or the shift in pH optimum of the proteinases of clostridia which results from the growth of these organisms in media of varying reactions (Kocholaty and Hoogerheide, 1938; Kocholaty and Weil, 1938). Although these changes exhibit a progressive course and are of a quantitative rather than of a qualitative character, there are indications that, here again, adaptation is due to the selec-

tion of variant forms rather than to the direct effect of the environment of the cell.

A few specific examples of increased resistance to toxic substances and of the biochemical reaction involved in this phenomenon will be presented in the discussion of drug fastness (Chapter VIII:5). It may be mentioned at this time, however, that the adaptation of *E. coli* to a new saline environment (increased concentration of sodium chloride) has been analyzed in terms of two components, namely, an acclimatization which does not involve reproduction and which has no hereditary basis or consequence, and a selection of the individuals endowed "with the widest range of potentialities" (Doudoroff, 1940).

In the case of higher organisms, temperature adaptation seems to be related to the degree of saturation of the cellular phosphatides which in turn conditions the melting point of these cellular components (Belehradek, 1935; Fraenkel and Hopf, 1940). It is possible, therefore, that even those variations which exhibit a progressive and only quantitative character are also the expression of stepwise chemical modifications, occurring normally as discontinuous variations in the parent culture, and selected under certain environmental conditions. Thus could be explained the "Dauermodifikationen" so common in microorganisms, and which so often gives the appearance of a transmissible effect of the environment on the cell.

Variations Induced by the Environment but Not Specifically Related to It.—Although many examples of bacterial variability deal with situations where the medium seems to exert a specific influence which favors the modified cell, there are other cases of hereditary variations which are apparently induced by environmental factors, but which do not bear any obvious specific relation to them. Thus, growth in the presence of injurious agents often results in nonspecific transmissible modifications. *E. coli*, for example, loses the ability to produce gas from sugars when grown on monochloroacetic agar and this change is associated with the development of small secondary colonies which occur on the surface of the large forms (Penfold, 1911). Rough colonies con-

sisting of long filamentous forms are produced when *Clostridium welchii* is grown in the presence of phenol (Habs and Mohr, 1935). Pneumococci change from the encapsulated to the nonspecific form in media containing bile acids and these substances also accelerate the production of avirulent variants of the tubercle bacillus. Antibacterial agents as chemically unrelated as barium chloride, gentian violet, penicillin, and gramicidin cause the production of small colony variants of the staphylococci which are resistant to many antiseptics (Chapter VIII:5). Nor need the conditions be unfavorable to produce these nonspecific variations. *Clostridium histolyticum* transplanted in protein media gives rise to colonies of irregular shape consisting of filamentous forms, whereas the original character of the culture can be regained by prolonged growth on protein-free media (Hoogerbeide, 1937).

Changes in Antigenic Structure Induced by Specific Antisera.—From the point of view of the immunologist, one of the most interesting modifications induced by the environment is the change in antigenic structure brought about by growth in immune antiserum. It has been repeatedly shown that pneumococci growing in media containing the homologous specific anticapsular antibody change more or less rapidly to the nonspecific, noncapsulated growth phase (Griffith, 1923; Reimann, 1925, 1927; Stryker, 1916). The nonspecific, noncapsulated form of *Klebsiella pneumoniae* can readily be produced by a similar technique (Julianelle, 1926) and the method also applies to the conversion of the smooth O forms of the salmonella into the rough nonspecific variants (Arkwright and Pitt, 1929). The converse change from the nonspecific to the specific form has also been obtained by growth in serum directed against the nonspecific phase; pneumococci are thus changed from the noncapsulated to the mucoid state (Dawson, 1928). Interchange in both directions has also been obtained by growing in their appropriate antisera two variants of *B. subtilis*, one motile and forming typical smooth colonies, the other non-motile and consisting of long chains forming medusa head colonies (Soule, 1928).

The changes induced by antisera exhibit great selectivity, de-

terminated by their immunological specificity. Thus, a serum directed against the S form of *S. enteritidis* (Gaertner), an organism which possesses antigen IX in common with *Eberthella typhosa*, is also capable of causing the latter organism to change from the S to the R form. When, however, the serum is heated at 70° C., or absorbed with the former culture in order to destroy or remove the specific antibody, it loses at the same time its ability to induce transformation of either organism (Scott, 1926).

As already mentioned, growth in homologous antiserum has been used to modify or completely suppress the H antigens of flagellated species. It must be remembered, however, that the flagellar antigens of many cultures spontaneously undergo those changes in antigenic specificity already referred to as phase variation (Chapter II:5 and IV:2). If repeated plate cultures are prepared from a strain in the specific flagellar phase, bacilli in the nonspecific group phase appear sooner or later in the culture. Similarly, a strain in the group phase gives rise to the specific phase by continuous cultivation (Andrewes, 1922). By growth in homologous antiserum, on the other hand, it is possible to reveal the existence of a second, hitherto unsuspected flagellar specificity in certain strains known under natural conditions only in the monophasic form, *i.e.*, with only one flagellar antigen. This has been achieved, for instance, with *E. typhosa*, and the artificial phase culture can be reverted to the typical monophasic form. Thus, the variation from one flagellar phase to another can occur in either direction, and the ready reversibility of the reaction contributes greatly to its interest, especially since it can proceed in cultures isolated from single cells (Simizu, 1938).

In other words, growth of an organism in the presence of an antibody specific for the flagellar antigen which it possesses inhibits the production of this antigen in the newly formed cells, and elicits in some cases the appearance of the other flagellar constituents which the culture is potentially capable of producing. Organisms which appeared to be permanently in the specific phase have thus been induced to change to the group phase, which then also seemed to be permanent until the organisms were again trans-

formed into the specific phase by growing them in group serum (Bornstein, 1943; Bruner and Edwards, 1939, 1941; Gard, 1938; Kauffmann, 1936c; Scott, 1926; Wassen, 1930). The experimental

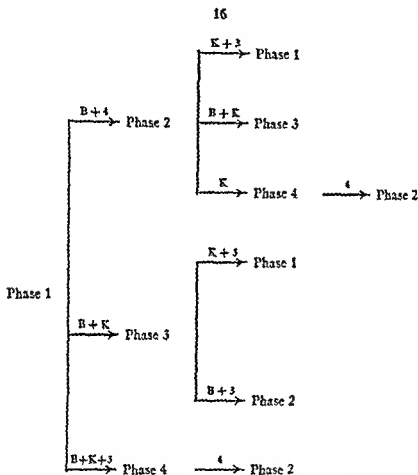


FIG. 24.—Changes induced in *S. paratyphi* A 228. Symbols on arrows indicate what serums were added to medium: B, serum derived from phase 1 of Bispebjerg type, K, serum derived from *S. cholerae* suis var. *kunzendorf*; 3, serum derived from *S. paratyphi* A, phase 3. The serum was absorbed with phase 1 before use; 4, serum derived from *S. paratyphi* A, phase 4. The serum was absorbed with phase 3 before use. (From Bruner and Edwards, 1941, fig. 1, p. 472.)

transformation of *S. salinatis* into a culture which cannot be differentiated from *S. sandiego*, illustrates the interest of the method.

When *S. salinatis* containing flagellar antigens d, e, h-d, e, n, z₁₅ is grown in the presence of a serum containing an antibody active against the d component, one obtains a new culture deficient

in this component and possessing the flagellar formula e, h-e, n, z₁₅. As already stated, the new culture thus artificially produced is identical in all respects with *S. sandiego*, a strain which had been isolated from a natural source. This transformation of one naturally occurring type into another in the laboratory gives an insight

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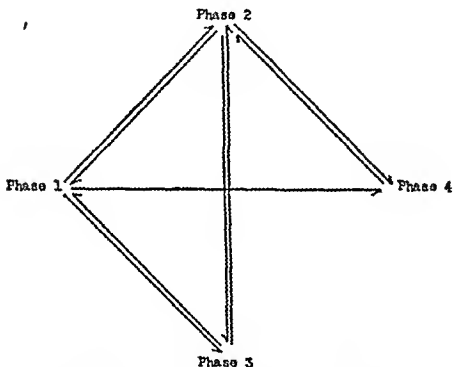


FIG. 25.—Reversibility of phases of *S. paratyphi* A 228. Arrows indicate direction in which variation occurred. (From Bruner and Edwards, 1941, fig. 2, p. 473.)

into the many changes in antigenic structure which are probably constantly taking place in nature. It suggests that the immunological complexity of the salmonella is due to the fact that the members of this bacterial group are in an unstable state and are undergoing evolution at a rapid rate (Edwards and Bruner, 1942; White, 1926, 1929b).

Any interpretation of the changes in antigenic structure induced by appropriate antisera must take into account the fact that, in many cases, the same changes can occur under other unspecific influences or even spontaneously without any detectable influence of the environment. When, for example, smooth strains of typhoid or paratyphoid A bacilli are subcultured in different media, it is found that R forms are produced most rapidly (ten days) in media containing immune sera directed against the S forms. R forms also appear, however, in other media devoid of specific antibody, although the change in these cases required many weeks or even months (Scott, 1926). Phase variation of the flagellar antigens is also a normal occurrence (Andrewes, 1922). Further analysis of these phenomena is therefore required to establish the mechanism of action of the antibodies, but it appears possible that these reagents only accelerate a process of change which goes on continuously in any culture, and that their action may consist only in a selective inhibition of the forms with which they react immunologically.

Variations Induced by Radiations.—The similarity, at least in appearance, of the discontinuous variations which affect the morphological, biochemical and physiological characteristics of bacteria, with the phenomenon of mutation in higher organisms, has stimulated attempts to cause bacteria to undergo variation by irradiation. In fact, production of filamentous cultures has been obtained by irradiation of bacteria with β and γ rays (Spencer, 1935); treatment of *Brucella abortus* with u.v. radiation at 2500 Å, causes a change from the S to the R form, although the R to S change could not be produced by this technique (W. Braun, 1943). Loss of ability to produce the lethal factor, the dermonecrosin, or the hemolysin can also result from x-ray treatment of staphylococci (Haberman, 1941). There is, of course, no indication that these modifications result from an effect of radiation on the nuclear mechanism and, in the absence of adequate knowledge, it would be hazardous to interpret them in terms of the similar phenomena observed in plant and animal cells.

5. MECHANISM OF TRANSMISSIBILITY OF HEREDITARY VARIATIONS

Apparent Correlation Between Different Characters.—Only scattered information is available to establish whether the many types of discontinuous variations which can effect a single culture and modify its morphological, biochemical, and physiological characters do occur as haphazard, unrelated events, or whether some kind of correlation exists between them.

The passage from one colonial form to another is sometimes accompanied by modifications of a number of other properties of the culture, a situation which might be interpreted as the manifestation of linked characters. In some lactobacilli, for example, the loss of ability to ferment sorbitol and mannitol is correlated with the loss of ability to produce the polysaccharide responsible for specific agglutinability (Harrison, 1942). A certain strain of *Staphylococcus aureus* capable of attacking only natural proline becomes active against both isomers when it changes to the rough white variant form. Continuous cultivation of cholera vibrios (obtained from single cells) in various suitable media gives rise to variants exhibiting new cultural, chemical, and serological characteristics; and these changes appear not to occur at random but on the contrary to be correlated (Linton, Seal, and Mitra, 1938). In the case of five different strains of *E. coli mutabile*, colonial dissociation invariably occurs concomitant with the metabolic variation resulting in rapid fermentation of lactose (Hershey and Bronfenhrenner, 1936). Passage of *Lactobacillus plantarum* from the S to the R forms is associated with loss of ability to produce acid from glucose, arabinose, lactose, and raffinose. In fact, whereas the R form can grow in sugar-free peptone, the S form does not grow in the absence of carbohydrates. In this case, therefore, the dissociation of S to R causes a saccharolytic bacterium to change into a nonsaccharolytic type, possessing enhanced ability to utilize nitrogenous nutrients (Tracy, 1938).

Before concluding that these associated changes constitute true manifestations of linked characters, it is, of course, necessary to

establish that their simultaneous occurrence is a reproducible, and not a chance, phenomenon. The possibility must be considered, furthermore, that the correlation is only apparent and that the different phenomena observed are only the multiple expressions of a single change in the cell. Thus, if a given enzyme capable of fermenting a certain sugar is an essential component of the system responsible for the production of a serologically active polysaccharide, sugar fermentation and immunological specificity will appear as two linked characters although they are only two different expressions of the same factor. Let us consider, as another example, an encapsulated culture of pneumococcus fully virulent for the mouse. This culture agglutinates in homologous antiserum and its colony possesses a characteristic glistening surface. Loss of the property to produce the capsule is accompanied by simultaneous changes in a number of other properties: the culture is no longer specifically agglutinable in homologous antiserum; it gives rise to colonies characterized by an uneven surface; it is no longer virulent for the mouse. Although capsule formation, specific agglutinability, mucoid character of the colony, and virulence are therefore correlated, these properties are not true examples of linked characters, since they are all the expression of only one character, namely, the ability to produce the type specific capsular polysaccharide (Chapters IV:4 and VI). This substance is the single component of the capsule; it determines agglutinability in specific antiserum, it probably accounts for the glistening aspect of the colonial surface; it conditions virulence by inhibiting phagocytosis. Similarly, the change in colonial morphology which occurs when cultures of the typhoid bacillus pass from the S to the R phase is also associated with decrease in virulence, loss of agglutinability in specific O antiserum, loss of stability of the cell suspension in saline solution, etc., and in this case these changes can be explained in terms of a single change, namely, the loss of ability to produce the somatic O polysaccharide antigen, a substance present in the S but absent in the R forms, and which conditions agglutinability and virulence (Chapters IV:2 and VI).

Independent Variation of Characters.—In fact, the majority of discontinuous variations which have been observed in bacterial cultures are examples of variation of independent factors (Nungester, 1933). Thus, on continued cultivation in artificial media, certain hemolytic streptococci lose the power to oxidize lactic acid while retaining their pathogenicity and the ability to oxidize pyruvic acid (Barron and Jacobs, 1938). The lactose positive variants of *E. coli mutabile* are culturally and serologically often indistinguishable from the parent strain (Hall, 1935; Hershey and Bronfenbrenner, 1936). A strain of *Shigella paradysenteriae* Sonne was found capable of giving rise to seven variants differing from each other in their capacity to ferment lactose, sucrose, and raffinose. Each variant remained identical with the parent organism in its specific agglutinability and other biochemical characters, and the fermentation characters remained unchanged when the culture underwent S \rightarrow R dissociation (Sears and Schoolnik, 1936). From one strain of *Bacillus pyocyaneus*, variants can be obtained which produce only the blue pigment, or only the yellow, or both, or none (Legroux, 1935). *Bacillus mycoides* gives rise to asporogenous strains which exhibit reversible changes of R to S, yellow to white, etc. (den Dooren de Jong, 1933). Encapsulated pneumococci can undergo a type of variation which affects their growth requirements (temperature, pH, CO₂ and O₂ tension), and their rate of autolysis, and which is independent of the smooth to rough dissociation (Eaton, 1934, 1935).

It may be mentioned at this time that the mucoid (M) property is not, as sometimes believed, a more advanced expression of the smooth (S) character of a culture; M and S can vary independently of each other. In the group of Gram-negative bacilli, S refers to that phase of the culture which possesses the specific somatic O polysaccharide antigen, whereas the M phase is characterized by the production of another viscous polysaccharide which is antigenically different from the O substance. R colonies with a mucoid structure and consisting of cells surrounded by a capsule have been described in cultures of *Serratia*

marcescens (Reed, 1937), and of *Cl. welchii* (McGaughey, 1933). In other words, mucoid forms do not necessarily possess the specific somatic polysaccharide, and, on the other hand, many cultures in the smooth phase are not encapsulated.

The independent variation of pigment formation and colonial morphology is well exemplified by certain strains of *Micrococcus tetragenes*, which produce, during prolonged growth under a variety of conditions, mucoid, smooth, and rough colonies, exhibiting different types of pigmentation: yellow, white, pink, pink yellow, or brown. Classification by colonial appearance of the different variants revealed that practically every possible combination of these different characters could thus be obtained:

Mucoid yellow—Mucoid white—Mucoid pink—Mucoid pink yellow—Mucoid brown
 Smooth yellow—Smooth white—Smooth pink—Smooth pink yellow—Smooth brown
 Rough yellow— Rough pink—Rough pink yellow—Rough brown
 (Reimann, 1936, 1937). (fig. 26.)

Similarly, cellular morphology, mucoid aspect of the colony, production of somatic antigen and pigmentation vary independently of each other in *Serratia marcescens* (Reed, 1937). (Table 24, fig. 27.) Finally, let us mention again that flagellated species lose and regain the ability to produce flagella, independently of other changes in the structure and properties of the cell, and that there can occur in the very composition of the flagellar material those discontinuous and independent variations already referred to under the name of phase variation (Chapters II:5, IV:2, and V:4).

The fact that the different characters of the bacterial cell can vary independently of each other has provided useful tools for the study of certain problems of infection. For example, analysis of the role played in virulence and immunity by the H, O and Vi components of the typhoid bacillus has been greatly facilitated by the discovery of variant forms of this organism deficient in one, in any combination of two, or in all three of these antigens (Chapters VI and VII). It is also certain that there occur many types of variation which have not yet been identified in terms of the classical antigenic and biochemical characteristics and

TABLE 24

A LIST OF VARIANTS ISOLATED FROM SEVEN CULTURES OF *S. marcescens*

CULTURE NUMBER	COLONIAL AND COLOR VARIETIES	
2446	S nonmucoid	red, orange-red, white
	S mucoid	red, orange-red, white
	R nonmucoid	red, orange-red, white
	R mucoid	red, orange red, white
	Medusoid	red, orange-red, white
2293	S nonmucoid	red, white
	R nonmucoid	red, orange-red, white
	R mucoid	red-orange, white
1377	S nonmucoid	red, white
	R nonmucoid	red, white
2302	S nonmucoid	red, white
	R nonmucoid	red, white
3804	S nonmucoid	red, white
2842	S nonmucoid	pink, white
	R nonmucoid	pink, white
air	S nonmucoid	red, pale pink, white

Data from Reed (1937, Table 1, p. 258).

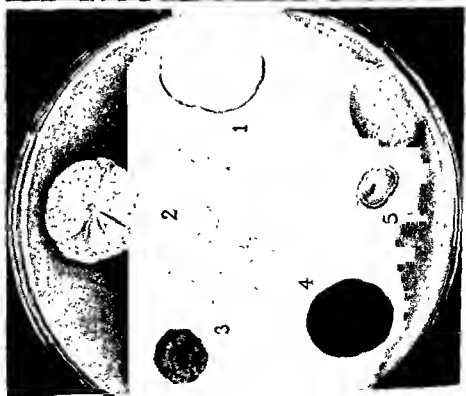
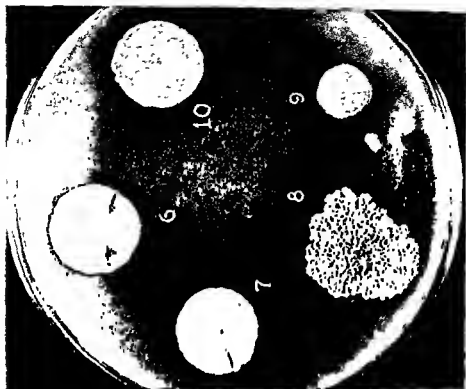
which affect the growth requirements and the metabolic products of an organism. These changes, although independent of immunological behavior, undoubtedly affect certain aspects of pathogenicity. The complex nature of virulence, and many obscure phenomena of epidemiology, will receive a satisfactory explanation only when more is known of the many types of variation which occur independently of the classical $S \rightleftharpoons R$ pattern and for which no morphological, biochemical or immunological test has yet been devised.

Frequency of the Occurrence of Variation.—Many of the early studies of variation have been carried out with cultures obtained by isolation of colonies, a method which gives no assurance that the colony has developed from a single cell and that the

culture is absolutely pure. There are on record, however, many examples of variation observed in cultures issued from single cells and affecting sugar fermentation, antigenic structure and virulence, sporulation and pigmentation, resistance to antiseptics, etc. (Barber, 1908; Duff, 1937; Kahn and Schwarzkopf, 1932; Simizu, 1938, 1939; Takahashi, 1938). It is clear, therefore, that any theory of the mechanism of variation must account for the fact that the ability to vary resides in the single vegetative cell and even in the endospore.

Analysis of the mechanism of variation is rendered more difficult by the paucity of quantitative data concerning the frequency of occurrence of the phenomena, a fact due in part to the impossibility of carrying out statistical studies within a bacterial population in which the different individuals cannot be readily differentiated. As already mentioned, any normal culture of *E. coli mutabile* contains 1 out of 100,000 cells which is endowed with the property to ferment lactose, and a similar rate of "mutation" has been observed when the quantitative aspects of the $S \rightleftharpoons R$ variation have been assayed by means of specific bacteriophage (Burnet, personal communication).

Quantitative studies of variation are somewhat more satisfactory in the case of bacterial cultures sufficiently unstable to give rise to a large and predictable number of variant forms. Thus, certain strains of *Salmonella aertrycke* have the property of constantly giving rise to two different colonial forms in detectable and predictable proportions (Deskowitz, 1937). The unstable bacterial culture appears as a mixture of two types of cells, those breeding true, *i.e.*, yielding a pure stable culture, and those constantly giving rise to the mixed culture. The terms "pure" or "stable" are employed in this connection in a relative rather than absolute sense. They imply that if the "stable" cultures exhibit variation, it is so rarely as not to be detectable under ordinary cultural conditions. As long as environmental factors remain unchanged, the ratio of the variant to the parent type was found to remain constant despite repeated selection of the latter, this ratio being, for instance, 41 per cent with a certain form (M), 19 per



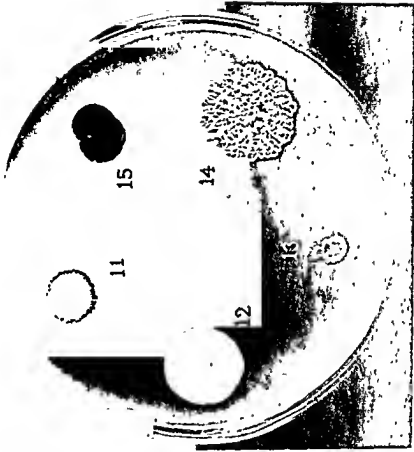


FIG. 26.—Photographs, slightly reduced, of point colonies on deep agar plates seeded simultaneously with 15 variant forms and incubated for 17 days at 25° C. 1 Mucoid-yellow, 2. Smooth-yellow, 3 Mucoid-white, 4 Smooth-white, 5 Translucent (the extra colony is a contaminant), 6. Mucoid-pink, 7. Smooth-pink, 8 Rough-pink, 9. Mucoid-pink-yellow, 10. Smooth-pink-yellow, 11 Rough-pink-yellow, 12 Mucoid-brown, 13. Smooth-brown, 14. Rough-brown, and 15 Bacillary colony. The rough-yellow colony is not shown, save for the pigment, it resembles that of the rough-pink colony (8). Sector formation may be noted in the smooth-yellow (2), mucoid-pink (6), and smooth-pink (7) colonies (From Reimann, 1937, fig 1, p 501)

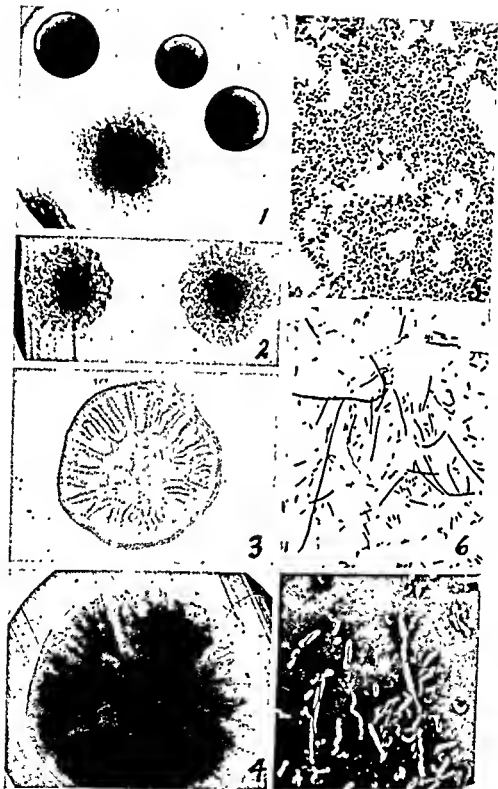


FIG. 27.—1 to 7. Photographs of colonies and organisms, *S. marcescens*
 1. S and R colonies. 2 R colonies 3 Medusoid colony. 4. S colony showing
 radiating bands of red and white pigmentation. 5. Organisms from a non-
 mucoid S colony. 6. Organisms from non-mucoid R colonies 7. Organisms
 from a mucoid R colony. (From Reed, 1937, plate, p. 256.)

cent with another one (R_3), and less than 2 per cent with a third (R_4). Altered environment, in certain instances, produced a temporary change in the ratio characteristic for each form, but on reculturing in the original environment, the ratio always reverted to its original value. The 2 per cent ratio, characteristic of form R_4 , was very close to the lower limit at which it was possible to recognize an unstable organism with the method used. It is apparent, therefore, that a so-called pure or stable organism could really be unstable and capable of undergoing variation, but that the percentage of variants given off could be so small as to escape detection during ordinary cultivation (Deskowitz, 1937).

Bacterial Variability and Classical Genetics.—These facts suggest that bacterial variation is based upon a fixed mechanism and that each organism has an inherent potentiality for producing a variant form once in a given number of divisions. Analysis of these phenomena in terms of classical genetics presents, however, many difficulties. Except in a very few suggestive cases, such as the production of mucoid material by paratyphoid B bacilli (Hage, 1925), and the production of pigment by *Serratia marcescens* (Marchal, 1932), there is no evidence that bacterial variation behaves according to Mendelian laws. Furthermore, knowledge of the structure and behavior of the bacterial nucleus is still inadequate, and despite many contrary claims, most workers still consider simple binary fission as the only method of cellular division in bacteria.

In the absence of some process of fusion and segregation, it is difficult to account at the present time for the observed phenomena of bacterial variation, whereas granting the assumption of the occurrence in bacteria of genetic impurity with some mechanism of segregation, the data of variability might fall in line with modern genetical conceptions. True evidence of fusion would open the whole field of problems originating in syngamy and amphimixis, and would permit the interpretation of variation in terms of combination and segregation. Production of variants would then be examples of segregation from genetically complex

parentage, while there would remain the additional possibility of the occurrence, at unpredictable intervals, of true mutations not depending upon segregation and recombination (Brierley, 1929, 1931). In the absence of convincing evidence, one can only mention a few of the many theories which have been advanced to explain the fundamental nature of bacterial variation. The phenomenon has been considered by some as essentially analogous to mutations in plant and animal cells (Baerthlein, 1918; Beijerinck, 1912; Dobell, 1913; Lindegren, 1935; Masini, 1907; Neisser, 1906). Other workers postulate an asexual character segregation (Reed, 1933; Stewart, 1927). Still others have seen evidence that conjugation (chiefly autogamous) can occur, often as a part of a complex life cycle (Almquist, 1924; Enderlein, 1925; Löhnis, 1921; Mellon, 1917, 1926, 1942). All these theories receive support from the claims that, in addition to vegetative multiplication by fission, bacteria can reproduce by conjugation, that they normally go through a complex life cycle, and that they are only one developmental stage in the life history of complex organisms.

6. LIFE CYCLE OF BACTERIA

Modes of Reproduction.—In the course of their normal growth cycle, bacteria undergo a progressive, orderly modification of many of their morphological, biochemical and physiological characteristics (Chapter V:2). In addition to these modifications, bacterial cultures also exhibit a number of abnormal forms which have been interpreted as being of significance in a more complex developmental cycle. Thus, old cultures often contain swollen bodies usually considered as involution forms, the production of which is stimulated by the addition to the medium of small amounts of certain substances such as magnesium sulphate, lithium chloride, phenol, etc. Although these forms are usually regarded as dying cells undergoing degeneration, it has been claimed that some of them are viable and represent a method of reproduction different from simple binary fission. Small bodies formed in, and

liberated from, these abnormal bacterial cells have been taken for gonidia capable of developing into bacterial forms typical of the species. Furthermore, the presence in microscopic preparations of cells in contact, or of cells which have apparently fused, has been considered as evidence of conjugation; cells with swollen portions in the middle being regarded as zygosporos resulting from fusion of two cells. The occurrence of two different types, particularly of large and small cells, has been taken as evidence of sexual differentiation into male and female forms. It has also been claimed that, at times, a whole group of bacteria can fuse to form an amorphous mass called symplasm. Within this mass there would appear minute deeply staining bodies of the nature of regenerative granules which in time can grow into recognizable bacterial forms. "All bacteria live . . . alternately in an organized and in an amorphous stage. By the partial or complete dissolution of the vegetative and reproductive cells, a plasmatic mass, the symplasm, is formed, which after a period of rest . . . may transform itself into new cells of the same or of a more or less modified character" (Löhnis, 1921).

The literature concerning these many types of hypothetical reproductive mechanisms is so controversial, and the claims so often based upon isolated observations and upon unspecific cytological techniques, that it seems unprofitable to attempt to evaluate the findings at the present time. We shall consider only a few specific cases in which the doctrine of life cycle has been subjected to more systematic investigation.

Significance of the L₁ Forms.—It has been repeatedly shown that all available cultures of *Streptobacillus moniliformis* contain a filter-passing pleuropneumonia-like organism, termed L₁, which can be isolated in pure culture and maintained in continuous subculture in a stable form (Klieneberger, 1935). Two hypotheses have been offered to account for the relationship between the two organisms (Sabin, 1941). According to some authors, L₁ is an independent microbial species which, under natural conditions, happens to be found associated and to live in symbiosis with *Streptobacillus moniliformis*. Indeed, it is possible by re-

peated isolations to obtain strains of L_1 which have been maintained over 100 to 300 passages without reversion to *S. moniliformis*; furthermore, a pleuropneumonia-like organism, morphologically, culturally, and immunologically identical with L_1 , has been isolated on at least one occasion from the lungs of a rat not containing *S. moniliformis*. The concept of symbiosis has been challenged by other investigators who believe that the L_1 form is not an independent microbial species, but rather a variant form of *Streptobacillus moniliformis*, a phase in its life cycle (Dienes, 1939). This belief is based on several facts. Strains of L_1 isolated and carried in pure culture on solid media revert to *S. moniliformis* in liquid media; the morphology of the L_1 organism does not differ materially from that of bacteria; the two organisms exhibit immunological relationship (Dawson and Hobby, 1939).

Both living and stained preparations of the L_1 organism show large swollen bodies containing great numbers of small coccus or rod-like structures which exhibit the staining properties of chromatin. It is claimed that these chromatin granules can germinate, and are a stage in the reproductive cycle of the pleuropneumonia organism and of the streptococcus. The large, swollen bodies, full of chromatin granules, have been seen not only in the L_1 form, but frequently in other pleuropneumonia organisms, and occasionally in cultures of some true bacteria. Furthermore, strains of the pleuropneumonia group have been isolated, not only from *Streptococcus moniliformis*, but also from cultures of *Bacteroides funduliformis*, *Haemophilus influenzae*, *Proteus vulgaris*, *Escherichia coli*, etc. (Dienes, 1939, 1941, 1942, 1944; Dienes and Smith, 1942). Indeed, many workers have described in various bacterial species the occurrence of giant cells assumed to arise as the result of a sexual process, and to represent a normal stage in the life cycle (Nyberg, 1938; Smith, 1944; Wahlin and Almaden, 1939). These findings have led to the generalization that all the pleuropneumonia forms are a special phase of a complex life cycle of bacteria which is characterized by a reproductive mechanism (the chromatin granules of the large bodies) similar to the gonidia of higher forms.

Life Cycle of Rhizobia.—Students of the root nodules caused by *Rhizobium leguminosarum* noted very early the presence of hypha-like infection threads of bacteroids within the root tissue, and they generally regarded these bacteroids as spores produced by a budding mycelium. Subsequent studies of the life cycle of rhizobia progressively led to the belief that the organism can reproduce by gonidia and even by symplasm and conjugation, and it has been generally accepted during recent years that the complete life cycle involves at least five types of cells occurring in the following sequence. Banded rods liberate small nonmotile cocci which can, however, become flagellated, and which increase in size to form larger nonmotile cocci. These elongate to form small ovoid motile cells and by further development change to elongated, unbanded, motile rods. The stainable substance rounds off to form small cocci, thus completing the cycle. External conditions influence the formation of cocci within the rods and the change to the motile condition, but the course of development was regarded as inherent and normal (Thornton, 1930).

More recent studies have cast doubt on the validity of the life cycle theory and indicate that it is based on erroneous interpretation of cytological observations. Specific cytochemical techniques have revealed that the "banded" condition is not caused by free cell formation, but by the deposition of unstainable fat bodies which restrict and compress the cytoplasm from the stainable bands (fig. 4). Furthermore, the small cocci and ovoid cells seen in old cultures are small vegetative cells caused by fission during the period of declining growth (Chapter II:1). What had been taken as evidence of reproduction by gonidia and of an orderly succession of different bacterial forms is then only the expression of aging and of pathological changes of the cell (Lewis, 1938, 1941).

Diphtheroid Phase of Streptococci.—It has also been claimed that certain streptococci exhibit a diphtheroid phase as a part of their normal developmental cycle (Mellon, 1917). There is indeed no doubt that many streptococci form diphtheroid-like cells in certain media and under the proper experimental conditions.

Whether these forms are the expression of a fundamental life cycle, or only the direct and reversible effect of the environment, has, however, been less clearly shown. Thus, it has been found that a strain of alpha-hemolytic streptococcus isolated from a case of subacute endocarditis readily assumes a diphtheroid appearance under the adverse conditions of extreme aerobiosis. Any toxic influence retarding the rate of division more effectively than the rate of protoplasmic synthesis would naturally result in the production of rod shaped organisms. Moreover, since the normal streptococcus does show transverse bands of protoplasm, it is obvious that the diphtheroid appearance can be the result of the immediate effect of external circumstances and not the expression of a developmental cycle (Lamanna, 1944).

The fact that, even in the case of the most thoroughly studied bacterial groups, cytological studies can lead to such conflicting interpretations makes it difficult to form an opinion concerning the existence of complex life cycles and of reproduction by gonidia. In fact, it is unlikely that these problems can be solved by cytological methods alone. In order to establish in a convincing manner the role played by a certain structure in some hypothetical development cycle, it will be necessary to develop methods for the production at will of this structure, for its separation from the bacterial culture, and for the study of its germination into new bacterial forms.

Sexual Reproduction in Bacteria.—The claims that bacteria can reproduce sexually, either by isogamy or heterogamy, rest on isolated observations, recorded as microphotographs, of cells in contact and apparently undergoing conjugation. They also receive support from the interpretation of the phenomenon of variation in terms of a rearrangement of chromatin by a sexual process. It is also the interpretation of variation data, rather than microscopic evidence, which has led to the view that asexual reproduction eventually comes to a close in a colony of bacteria and is followed by an "outburst of conjugation" consisting in an autogamous process, a theory which appears to be substantiated by cer-

tain observations on chromosome behavior (Allen, Appleby, and Wolf, 1939; Badian, 1933; Stewart, 1927).

If bacteria do really reproduce by sexual methods, it should be possible to cross closely related species and strains and to determine something of their genetical behavior. Although there have been isolated reports of successful crossing, most workers who have attempted to cross related strains have reported only failure (Gowen and Lincoln, 1942).

In effect, the evidence presented to establish sexual reproduction in bacteria is not convincing. Mere fusion of cells does not necessarily mean sexual reproduction since this process implies a union of chromatin from two individuals. In fungi, for example, cells may be seen to fuse without any evidence of nuclear fusion and without formation of a reproductive body. The occurrence of transmissible variations cannot be used either as evidence of hybridization and sexual reproduction, especially since it has not yet been proven that the inheritance of characters in bacteria follows the Mendelian formula. It may be recalled in passing that sexual reproduction has not been proven either in the Cyanophyceae, despite the fact that these plants are larger than bacteria and more favorable for cytological study.

The Significance of Bacterial Dissociation.—Although the changes in colonial morphology (M, S, R, D, G, etc.) associated with modifications of the antigenic structure have been discussed in preceding pages as examples of discontinuous variations, there are many authors who regard these manifestations of bacterial variability as normal stages in the developmental life cycle of the individual organism (Hadley, 1927, 1937, 1939). "Any bacterial culture, arising from a single cell and developing on common culture mediums, may manifest in the course of time many diverse cell forms and culture growths. Those most frequently observed are the mucoid, smooth and rough, while a gonidial phase (G phase) and a diphtheroid (D phase) are present in several species." "What might be considered a sort of ontogeny for the individual of any bacterial species would comprise pro-

gressive development through the culture phases G, S, and R. Morphologically, they begin as the tiniest of coccus forms (the G) and progress to the coccus, chained, and filamentous structures; the latter being characteristic of the rough phase culture in all families above the Coccaceae. An interesting parallel exists with reference to the great so-called family groups of bacteria. The progression of the micrococci to the Coccaceae, to the Bacillaceae, to the Bacteriaceae—the Mycobacteriaceae—the Actinomycetaceae—etc., exemplifies it." Thus Haeckel's aphorism that ontogeny recapitulates phylogeny would also apply to bacteria, and the different forms in which we know bacteria, smooth, rough, etc., would be only more or less permanently stabilized cyclogenic ontogenic phases of polyphasic organisms (Mellon, 1926).

Variation and the Problems of Taxonomy and Classification.—The property of the bacterial cell to exist and become stabilized in a multiplicity of forms adds new difficulties to the problems of taxonomy and classification (Hadley, 1939). Under the influence of the doctrine of monomorphism, one so called "normal" aspect of a given culture has been selected for description. There are unfortunately no criteria to define the "normal" aspect of a culture, and in practice bacteriologists have selected as normal the forms which most readily become acclimatized to the standard laboratory conditions. Moreover, for a number of often irrelevant reasons—chiefly on historical grounds—the forms which are considered "typical" or "normal" vary from one species to the other. In the case of pneumococcus, for example, the mucoid phase is described as typical, and in the case of typhoid bacillus, the smooth phase is selected. These organisms can, however, exist in a number of other forms and there is no evidence that, from the point of view of the biology of the bacterium, *i.e.*, the only point of view of importance for taxonomy and classification, the rough phase of the pneumococcus is less important than the mucoid, the mucoid form of the typhoid bacillus less normal than the smooth. The problem becomes even more complex, although it would become simpler and clearer with more complete knowledge, if bacteria are really the imperfect forms of organisms which

can under some conditions give rise to a sexual stage. If this is true, any attempt to classify bacteria in the present state of ignorance is likely to duplicate the confusion which prevailed over the group of *fungi imperfecti* before their sexual forms were identified.

Need of New Experimental Methods.—It is obvious that an accurate knowledge of the modes of reproduction and of the life cycles of bacteria is not only the necessary basis of any system of classification, but that it would help to clarify some obscure aspects of the natural history of infectious diseases. It is regrettable, therefore, that so much of the information available on this problem rests upon isolated observations, and so little upon systematic experimentation. In fact, many of the descriptions of life cycles represent only attempts to patch together haphazard observations, often made in cultures growing in widely different media, without much regard to the age of the cell, and to the well established cellular modifications which occur during the growth cycle or as a result of changes in the environment.

Lack of direct, convincing evidence does not, naturally, invalidate the claims of the existence of a complex life cycle in bacteria. It was almost through fortuitous circumstances that the perfect forms of *fungi imperfecti* were discovered; dependable techniques for the production of the sexual forms in yeasts are a fairly recent development, and it is only now that the transmission of characters in these organisms can be analyzed in terms of the gene theory. As with certain yeasts and other fungi, sexual conjugation in bacteria may require highly specific conditions which the common bacteriological media do not usually provide. Moreover, failure to observe the germination of the small intracellular bodies which have been considered as gonidia, may be due to a number of causes: the intense light required for observation may be injurious, germination may take a very long time (as is the case with certain endospores), or it may require certain peculiar environmental conditions (Sander, 1938).

It is certain, however, that only slow progress can come from mere chance observations. New experimental methods are re-

mutation of types therefore reveals among the noncapsulated variants the existence of a number of forms endowed with varying potentialities and physiological abilities.

The substance which elicits the transmutation has been isolated from type III pneumococci in a highly purified form. Addition of 0.003 μ g of the purified fraction, to 2 cc. of an appropriate serum broth inoculated with 0.000,005 cc. of a "competent" non-capsulated culture derived from type II pneumococcus, is sufficient to convert the latter into encapsulated pneumococci of type III which retain thereafter the new specificity. In other words, the activity of the transforming principle can be recognized in a final dilution of 1:600,000,000. (Fig. 28.)

"The data obtained by chemical, enzymatic, and serological analyses together with the results of preliminary studies by electrophoresis, ultracentrifugation, and ultraviolet spectroscopy indicate that, within the limits of the methods, the active fraction contains no demonstrable protein, unbound lipid, or serologically reactive polysaccharide, and consists principally, if not solely, of a highly polymerized, viscous form of desoxyribonucleic acid. On the other hand, the Type III capsular substance, the synthesis of which is evoked by this transforming agent, consists chiefly of a non-nitrogenous polysaccharide constituted of glucose-glucuronic acid units linked in glycosidic union. The presence of the newly formed capsule containing this type-specific polysaccharide confers on the transformed cells all the distinguishing characteristics of *Pneumococcus* Type III. Thus, it is evident that the inducing substance and the substance produced in turn are chemically distinct and biologically specific in action and that both are requisite in determining the type specificity of the cell of which they form a part."

The experimental findings demonstrate "that the induced alterations are not random changes but are predictable, always corresponding in type specificity to that of the encapsulated cells from which the transforming substance was isolated. Once transformation has occurred, the newly acquired characteristics are thereafter transmitted in series through innumerable trans-

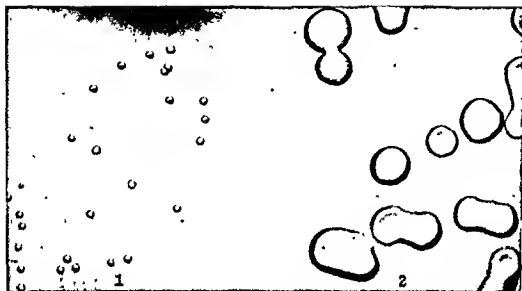


FIG 28—Transformation of pneumococcal types 1 Colonies of the R variant (R36A) derived from *Pneumococcus* Type II Plated on blood agar from a culture grown in serum broth in the absence of the transforming substance ($\times 35$) 2. Colonies on blood agar of the same cells after induction of transformation during growth in the same medium with the addition of active transforming principle isolated from Type III pneumococci The smooth, glistening, mucoid colonies shown are characteristic of *Pneumococcus* Type III and readily distinguishable from the small, rough colonies of the parent R strain illustrated in 1. ($\times 35$). (From Avery, Macleod, and McCarty, 1944, pl. 1, p. 158)

fers in artificial media without any further addition of the transforming agent. Moreover, from the transformed cells themselves, a substance of identical activity can again be recovered in amounts far in excess of that originally added to induce the change. It is evident, therefore, that not only is the capsular material reproduced in successive generations, but that the primary factor which controls the occurrence and specificity of capsular development, is also reduplicated in the daughter cells. The induced changes are not temporary modifications but are permanent alterations which persist provided the cultural conditions are favorable for the maintenance of capsule formation" (Avery, MacLeod, and McCarty, 1944).

If the transformation thus induced is described as a genetic mutation, it offers an authentic case of specific mutation brought about by a specific treatment, a feat which geneticists have vainly tried to accomplish in higher organisms (Dobzhansky, 1941; Sonneborn, 1943). Assuming that the substance which induces transformation is really a desoxyribonucleic acid, as the evidence strongly suggests, then nucleic acids of this type must be regarded not merely as structurally important, but as functionally active in determining the biochemical activities and specific characteristics of the pneumococcal cells; they possess a biological specificity the chemical basis of which is as yet undetermined. Of equal interest is the fact that, in order to respond to the stimulus exerted by the transforming principle, the bacteria must possess these obscure physiological properties which determine the state of "competence." Furthermore, transmutation requires that certain cultural conditions be fulfilled before it is possible to demonstrate the reaction even when a competent cell is placed in the presence of a potent preparation of the transforming principle. Recognition and control of these different factors which play a part in the reaction render the transmutation of pneumococcus types a predictable, reproducible phenomenon. Definition of their nature will eventually elucidate the mechanism of the transformation and will, one may hope, provide a pattern for the analysis of several of the phenomena of bacterial variability.

VI

THE NATURE OF VIRULENCE

"When I use a word," Humpty Dumpty said, in rather a scornful tone, "it means just what I choose it to mean—neither more nor less."

LEWIS CARROLL

... we are too much accustomed to attribute to a single cause that which is the product of several, and the majority of our controversies come from that.

JUSTUS VON LIEBIG

1. HOST-PARASITE RELATIONSHIPS

M*anifestations of Virulence.*—The term virulence was used in medical terminology long before the microbial theory of infection was established; it referred to the poisonous quality of an agent or served to qualify the severity of a disease or of an epidemic. With the advent of the bacteriological era, most of the characteristics of the infectious process were for a time referred to the etiological agent, without much regard to the host except in its response to infection through the reactions of immunity. Thus, by an unjustified extension of meaning, the term virulence came to be applied to a hypothetical property of the infectious agent which was assumed to determine the severity of the disease; the presence or absence of virulence was considered to be a well defined and intrinsic quality of the microbial species under consideration. The problem is in reality much more complex. Not only does the ability to produce disease vary from strain to strain within the same microbial species, but within the same strain it also varies with the state of dissociation of the culture and with its history, both *in vivo* and *in vitro*. Furthermore, a micro-organism highly pathogenic for a certain animal host is often harmless not only for hosts belonging to other species, but even

for other individuals of the same species or of the same strain. The biological nature and the physiological condition of the host are, in other words, fully as important as the nature of the parasite in determining the severity and the outcome of the infection (Th. Smith, 1934). We shall outline in the following pages some of the many factors involved in the manifestations of virulence in order to illustrate its complex nature. It will not be our purpose to consider the varied influences which modify the response of the host to infection, but rather to emphasize the components of the problem which directly concern the infectious agent.

In spite of the severity of the pathological process which it causes, *Clostridium tetani* does not invade the tissues beyond the point of initial lodgment. In fact, the tetanus bacillus is not usually capable of maintaining itself and of multiplying even at this point unless the tissues have been injured by some other agent. Thus, the condition which ensues is not properly an infection, but rather a toxemia. In this case, then, virulence is characterized primarily by the ability to produce a powerful toxin. The anthrax bacillus, on the contrary, tends to spread rapidly from the point of entrance to cause a general infection of the blood and viscera. Although this organism probably produces toxic substances, its virulence is primarily characterized by the amazing rapidity and luxuriance with which it multiplies in the susceptible hosts. The ability to invade or the property of toxigenicity are not always sufficient by themselves to enable an organism to cause disease. For instance, *Trypanosoma lewisi* can multiply and reach enormous numbers in the blood stream of rats without causing any obvious pathological reaction; a similar situation is sometimes observed with the spirochaete of relapsing fever in man. On the other hand, *Bacillus botulinus*, even though capable of producing the most powerful known toxin, does not infect man or animals; botulinus poisoning is almost always due to the toxin formed outside of the body during growth of the bacteria in material subsequently ingested.

In the septicemic infections caused by plague bacilli or hemolytic streptococci, the etiological agent invades the animal body

and multiplies abundantly in many different tissues. The infection is furthermore accompanied by a profound toxemia probably due to the fact that either the cellular constituents of these bacteria or their metabolic products, or both, are extremely poisonous. In these cases, the ability to invade and the ability to poison, both contribute to the virulence of the organisms. It must be emphasized, however, that even the combined ability to multiply *in vivo* and to produce toxin is not always sufficient to render a microorganism virulent for a given animal species under natural conditions. Thus, although strains of pneumococci or of group A streptococci are capable of causing a fulminating septicemia in mice or rabbits when injected by the intraperitoneal or intravenous route, they have never been known to cause spontaneous infection in these animals. Another attribute of virulence appears to be, therefore, the ability to become established in the host under natural conditions, a property which has been described under the name of communicability (Coburn, 1944; Coburn and Pauli, 1941; Schwentker, Janney, and Gordon, 1943).

Analysis of the different manifestations of streptococcus infections in man illustrates the complexity of the factors involved in virulence. A hemolytic strain of group A streptococcus may be highly invasive and cause bacteremia; it may produce a powerful erythrogenic toxin and cause intense scarlatina; one or more of the cellular constituents and products may produce severe febrile reactions and collapse of the host. Such highly pathogenic strains, however, may have a low degree of communicability; or, conversely, highly communicable organisms may produce little evidence of disease. All these factors vary independently of each other, and to be capable of causing severe epidemics, a strain must possess several or all of the properties which have just been considered, and probably others as yet unidentified (Coburn, 1944).

Enhancement of Virulence by Animal Passage.—Some species of microorganisms rapidly lose their virulence when propagated on artificial media. For example, three to six anthrax bacilli taken directly from the blood of a dead animal regularly cause the

death of mice, whereas the MLD is increased several fold when the infective inoculum is taken from a 12-hour agar culture and becomes very much larger after several generations on agar (Webb, Williams, and Barber, 1909). Conversely, repeated passage of a bacterial agent through a given animal species often results in a marked increase of virulence of this agent for this particular host. It is also claimed, without convincing evidence, that a similar increase in virulence of the pathogens can be observed in human infections at the beginning of an epidemic. The specific nature of the modifications which render the parasite more efficient in attacking the host is often obscure. In fact, so far, only one of the factors involved, namely, the change in dissociative state of the infectious organism, has been defined in clear terms.

As we have seen, the change in dissociative state of a bacterial culture expresses itself by many modifications of colonial and cellular morphology and of immunochemical specificity (Chapters IV:2 and V:4). This fact has facilitated recognition of bacterial dissociation and its correlation with changes of virulence. It is of special interest in this respect that, during the active phase of a disease, the infective agents are almost always present in the host in one particular form; thus the *Coccaceae* and the *Bacteriaceae* are isolated in the smooth or mucoid state from pathological material; the virulent forms of the anthrax bacillus give colonies which are rough although encapsulated (Hadley, 1939a). When an organism in a nonvirulent dissociative phase is injected into a suitable experimental animal, either it may be completely eliminated, usually by bacteriolysis or phagocytosis, or if it becomes established in the host, it usually undergoes a transformation into the dissociative phase which is characteristic of the virulent state. For example, the injection of large numbers of nonencapsulated pneumococci into mice may occasionally result in death of the animal, but when this happens encapsulated forms are isolated at autopsy. In addition to the change of dissociative phase, there often occur as a result of animal passage further modifications for which no morphological or immuno-

logical criteria have so far been recognized. Thus, the virulence for mice of the "matt" forms of group A streptococci can often be considerably raised by repeated passage through these animals, without any detectable modification of colonial morphology or immunological specificity.

These different types of modification of virulence are illustrated by the following observations. A strain of alpha hemolytic streptococcus of low virulence for mice was isolated from a human case of subacute endocarditis; it was transformed into the rough phase by cultural methods *in vitro*; the MLD for mice of the rough variant was 0.7 ml. In the course of 11 consecutive passages through mice, the organisms reverted from the rough to the smooth (or "matt") phase, the MLD simultaneously changing from 0.7 ml. to 0.005 ml., a degree of virulence approximately the same as that of the smooth culture on first isolation from the human material. The organism remained in the smooth phase from the 12th to the 38th mouse passage, while the virulence increased progressively to reach an MLD of 0.000.001 ml., without any detectable modification of any morphological or immunological property (Hadley and Wetzell, 1943). This type of increase in virulence which occurs above and beyond the change in dissociative phase may exhibit considerable specificity with reference to the animal species which induces it. It is most readily explained as resulting from the selection of those variant forms normally present in the parent culture which are best adapted to multiplication in the particular host under consideration (Chapter V:4). Increase of virulence for one given host can even be associated with decrease in virulence for another animal species.

Definition of Virulence.—It is clear that the ability of a micro-organism to establish a pathological state in a given host is the summation of a number of different and independent attributes such as communicability, invasiveness, toxigenicity, etc. The definition of virulence is rendered still more complex by the fact that an organism pathogenic for a certain host may be entirely innocuous for another. Thus, guinea pigs are often very resistant to infection with human strains of pneumococci, whereas

other rodents, such as mice and rabbits, can be readily infected with these same organisms by the intraperitoneal, intravenous, or intradermal route. There are enormous variations of susceptibility to the same infective agent even within the same animal species, as from one breed to another, and even from one individual to another depending upon the age, the physiological state, and a number of other undetermined factors in the host. Furthermore, the route through which the infective agent is introduced greatly modifies the results of the virulence test. Let us consider, for instance, a "highly virulent" culture of pneumococcus, capable of establishing a fatal disease in mice following the injection of one, or of a very few cocci, by the intraperitoneal route. When the same culture is introduced by the intravenous route, much larger numbers of cocci, often several millions, are required to establish the infection (Oerskow, 1940). Even more striking is the fact that attempts to introduce the infective agent by the intranasal route almost invariably fail, unless the general resistance of the host, and especially the anti-infectious barriers, have been weakened as by anesthetization with ether or alcohol (Rake, 1936; Stillman, 1938; Stillman and Branch, 1930; Stillman and Schulz, 1940; Webster and Clow, 1933). Similarly, an erysipelas strain of hemolytic streptococcus, which induced severe spreading lesions when injected intradermally into rabbits, was found to have no lethal power when injected intraperitoneally or intravenously, in even large doses (Rivers, 1925).

One could multiply *ad nauseum* examples illustrating the fact that virulence must always be qualified in terms of the particular host to be infected, and of the exact conditions under which the infectivity test is performed. Virulence is not a permanent, intrinsic property of a given species. It expresses only the ability of a given strain of the infective agent, in a certain growth phase, to produce a pathological state in a particular host, when introduced into that host under well defined conditions. This definition restores to the word virulence much of its earlier meaning; it refers to the disease and to the host-parasite relationship, rather than to some unique attribute of the microorganism. Complete

analysis of the problem of virulence would require, therefore, a description of the many microbial and host factors which affect infection and resistance. The following discussion will be limited to a brief statement of some of the properties of the bacterial cell which affect its relation to the host.

2. RESISTANCE OF THE PARASITE TO THE DEFENSE MECHANISMS OF THE NORMAL HOST

Antibacterial Mechanisms of the Normal Host.—Following their introduction into the animal body, microorganisms become subjected immediately to the action of the varied defense mechanisms of the host. Tissues and body fluids contain a number of ill defined and nonspecific substances, many of them heat stable, which exhibit some degree of bactericidal activity against certain bacterial species and which are described according to their origin under the names of β lysins (present in the serum), leukins (from the leucocytes), plakins (from the blood platelets), etc. (Topley and Wilson, 1937, page 518). Some of these bactericidal agents appear during the febrile period of a variety of diseases and have been shown to be particularly active against streptococci (Tillett, 1937; Wulff, 1934). So little is known of their nature, mode of action and importance, that it is hardly profitable to do more than mention them in the present discussion.

The bacteriolytic enzyme lysozyme is also commonly present in tissues and body fluids, and deserves some consideration at this time (Chapter IV:1). Even in high dilutions, this enzyme causes the death of bacteria of several saprophytic species. Since it is inactive against most pathogens, or at best only slightly active in high concentrations, it has been considered to be of no significance in antibacterial resistance. The possibility remains, however, that there exist a number of microorganisms which are potentially pathogenic and which are prevented from manifesting their pathogenic properties solely because they are susceptible to lysozyme and therefore cannot establish themselves in the animal body. It may be of interest to recall in this respect the

claim that certain strains of staphylococcus can be rendered more virulent by training them *in vitro* to become resistant to lysozyme.

The bacteria which find their way, or are injected, into a normal host are also exposed to the bactericidal or bacteriolytic effect of the natural antibodies acting in combination with complement. Little is known of the origin of these natural antibodies, whether they are produced by a normal physiological process, or are the result of unrecognized antigenic stimulation. In any event, it is certain that they are normally present in the serum of many adult animals and that, by reacting with the bacteria, especially those of the Gram-negative species, they prepare them, "sensitize" them for lysis by the complex system called complement, also a normal constituent of the body fluids (Gordon and Carter, 1932; Gordon and Johnstone, 1940, 1942; Topley and Wilson, 1937, page 851). In this case again, little is known of the actual occurrence and importance of complement bacteriolysis *in vivo*, and of its relation to normal immunity.

The process of phagocytosis is of great importance in ridding the body of bacterial invaders. Like other foreign particles, most bacteria can be engulfed by the wandering cells and by those of the reticulo-endothelial system (Metchnikoff, 1901). Phagocytosis is such a common biological phenomenon that the resistance of virulent microbial species to this process attracted very early the attention of the students of infection who observed, for instance, that whereas virulent streptococci injected into normal guinea pigs rapidly multiply without undergoing phagocytosis, avirulent proteus bacilli simultaneously injected are immediately taken up in enormous numbers by the leucocytes. Similarly, leucocytes resuspended in normal serum are capable of taking up nonvirulent streptococci, but are inactive against the virulent forms (Bordet, 1897; Marchand, 1898). The relation of virulence to phagocytosis has since been established in many other cases, and the factors which come into play in this relationship will be considered at greater length later (Chapters VI:2 and VII:3).

Relation of the Dissociative Phase of the Culture to Virulence.—Much of our knowledge of the nature of virulence has

been derived from the fact that every species of pathogenic micro-organism has yielded one or several avirulent variant forms. Variation in colonial morphology, cultural characteristics, and virulence was recognized very early in several species of bacteria (Baertlein, 1918). It is with the group of Gram-negative bacilli, however, that correlations between the morphological and biological differences of the rough (R) and smooth (S) forms received its first accurate statement (Arkwright, 1921; De Kruif, 1921, 1922; Schütze, 1921). It was found that cultures of *Past. leptisepticum* maintain their original virulence as long as they remain in the smooth phase, but that they become relatively avirulent when they change to the R form (De Kruif, 1921; Webster and Burn, 1927). Subsequent observations have confirmed and greatly extended this original finding, and there is little if any doubt that the S forms are responsible for the natural infections caused by the pasteurella, the salmonella, the shigella, the brucella, etc. It must be kept in mind, on the other hand, that R variants of *Shigella paradysenteriae* Sonne are often recovered from clinical dysentery in man, and that the R forms of the mouse typhoid bacillus retain to a certain extent the ability of establishing a fatal disease in experimental animals (Webster and Burn, 1927).

One of the most important cellular modifications associated with the S \rightarrow R variation in the Gram-negative bacilli is the loss of the type specific polysaccharide antigen designated as O, and it is natural to assume, therefore, that this antigenic component plays an important part in virulence (Chapter IV:2). The fact that antibodies specifically directed against the O antigens confer protection against infection with the corresponding smooth organisms, in both natural and experimental diseases, is good evidence in favor of this view (Chapter VII:2, 3). Additional suggestive evidence is found in the observation that preparations of the O antigens isolated in solution from the bacterial cells exhibit definite physiological activities which may be of significance in infection. Injection of these substances into normal animals causes a rapid and intense leucopenia, either directly

through the destruction of the leucocytes, or indirectly by causing their migration outside of the blood stream (Morgan, 1941, 1943; Munger, 1941; Olitzki, Avinery, and Bendersky, 1941; Olitzki, Avinery, and Koch, 1942; Robertson and Yu, 1938). It has also been observed that the O antigen of the typhoid bacillus exerts a repellent action against leucocytes and may thus interfere with phagocytosis (Morgan and Upham, 1941). Furthermore, the same toxic O antigens inhibit the bactericidal power of the serum against the homologous organisms, whether this bactericidal power is due to normal or to immune antibodies (Cundiff and Morgan, 1941; Thihault, 1939) (Tables 25 and 26).

TABLE 25

INHIBITION OF THE BACTERICIDAL ACTIVITY OF NORMAL RABBIT SERUM (0.1 CC) BY THE COMPLETE POLYSACCHARIDE ANTIGEN OF *Shigella dysenteriae* (SHIGA)

NUMBER OF BACILLI	TEST ORGANISMS						
	SHIGA BACILLI			FLEXNER BACILLI		DISS BACILLI	
	Amt of inhibitory Shiga extract (mg)			Amt of inhibitory Shiga extract (mg)		Amt of inhibitory Shiga extract (mg)	
	0	0.02	0.2	0	0.2	0	0.2
9 - 12 × 10 ¹	-	-	-	-	-	-	-
× 10 ²	-	-	++	-	-	-	-
× 10 ³	-	-	++	-	-	-	-
× 10 ⁴	-	-	++	-	-	-	-
× 10 ⁵	-	++	+++	++	++	-	-
× 10 ⁶	-	+++	+++	+++	+++	-	-
× 10 ⁷	+	+++	+++	+++	+++	++	+
× 10 ⁸	+++	+++	+++	+++	+++	+++	+++

- = no bacterial growth

+ - +++ = amount of bacterial growth

Data from Thihault (1939, Table vii, p. 474).

There is little doubt, therefore, that the O antigens, characteristic of the smooth variants of Gram-negative bacilli, protect these organisms against some of the defence mechanisms of the host and contribute thereby to the property of virulence.

TABLE 26

INHIBITION OF THE BACTERICIDAL ACTIVITY OF NORMAL AND ANTITYPHOID SERA AGAINST *E. typhosa* BY BACTERIAL EXTRACTS

INHIBITORY EXTRACT	NORMAL GUINEA PIG SERUM	ANTI- BACTERIAL RABBIT SERUM 1:400	ANTI- EXTRACT RABBIT SERUM 1:200	ANTI- EXTRACT HUMAN SERUM 1:512	ANTI- EXTRACT HUMAN SERUM 1:64
<i>E. typhosa</i>					
3 mg	+	+	+	+	+
1 mg	+	+	+	+	+
0.5 mg	+	+	+	+	+
0.1 mg	+	+	+	+	+
0.05 mg	0	0	+	+	+
<i>S. enteritidis</i>					
3 mg	+	+	+	+	+
1 mg	+	+	+	+	0
0.5 mg	0	0	0	0	0
<i>S. paratyphi B</i>					
5 mg	+	+	+	+	+
1 mg	0	+	+	0	0
0.5 mg	0	0	0	0	0
Salt solution	0	0	0	0	0
Serum control	+	+	+	+	+
Antigen control	+	+	+	+	+
Comp. and bact	+	+	+	+	+
Excess comp	•	•	•	•	•
Bacterial count/ml	4400	4090	3280	4360	3170

+ = growth; 0 = no growth

* Complete lysis of sensitized r b c occurred in tests with these mixtures showing free complement was present.

Data from Cundiff and Morgan (1941, Table 2, p. 364)

We have mentioned the existence in certain strains of the typhoid bacillus of another antigen Vi which is apparently a surface component of the cell and which, except in a few cases, is always found in cultures isolated directly from human infections (Almon, 1943; Félix and Pitt, 1934, 1935, 1936; Dolman, Kerr, and Helmer, 1941) (Chapter IV:2). As in the case of the

O antigens, the role of Vi in virulence has been confirmed by demonstrating that the antibodies specifically directed against it confer protection to animals experimentally infected with Vi strains (Almon, 1943; Boivin, Izard, and Sarciron, 1939; Grasset and Lewin, 1937; Henderson, 1939). If, as has been claimed, Vi is similar to the O antigens in general chemical composition, it may also possess their physiological activities and thus protect the organisms which contain it against the defense mechanisms of the host. This role may be especially important since Vi appears to be the most superficial constituent of the cell.

The phenomenon of bacterial dissociation has permitted the recognition in many other bacterial species of varied antigenic components of importance to virulence. It is sufficient to state that, among group A streptococci, all virulent strains have been found to possess a peculiar protein, the M substance, which confers type specificity upon the different strains (Chapter IV:2). The importance of the M substance has been demonstrated by the fact that immunization with preparations of it isolated in a soluble, purified form from the streptococcus cell, results in the development of active and passive immunity against experimental infections with virulent organisms of the homologous type (Hirst and Lancefield, 1939; Lancefield, 1941; Wiener, Zittle, and Mudd, 1942). No information is available concerning the physiological activities of the M substance which account for its role in virulence, except for the fact that it is a surface component of the cell (Lancefield, 1941, 1943).

Bacterial variability comprises many phenomena which are independent of the $S \rightleftharpoons R$ dissociation, and, for example, the variation to the M (mucoid) phase, characterized by the production of viscous material which accumulates around the cell as a capsule, is of special importance in the present discussion (Chapters II:4, IV:2). The relation of virulence to possession of a capsule by the invading organism was recognized very early (Bordet, 1897; Preisz, 1911; Stryker, 1916), and the subject has assumed such importance and has been so extensively studied that it will be considered at greater length in subsequent pages

(Chapters VI:2 and VII:2). It may serve to illustrate the complexity of the relationships of bacterial dissociation to virulence, however, to point out at this time that, in streptococci, both the type specific M proteins (corresponding to the matt & glossy variation) and the capsular polysaccharide, condition the ability of the organism to establish a pathological state. Furthermore, we shall emphasize repeatedly that many other attributes are required to confer full virulence upon type specific encapsulated strains (Table 27).

TABLE 27

DISSOCIATIVE PHASE AND VIRULENCE IN *Streptococcus hemolyticus* GROUP A

Morphology of colonies	Mucoid	Matt	Smooth (finely granular)	Rough
Morphology of organisms	Diplococci or short chains; uniform	Short chains; slightly pleomorphic	Short chains; uniform	Long tangled chains; large and coarse with extreme pleomorphism
Capsules	+	0	0	0
Growth in plain broth	Diffuse (or finely granular)	Granular	Diffuse (or finely granular)	Flocculent
Type-specificity	+ or 0	+ or 0	+ or 0	0
Virulence				
Human	+ or 0	+ or 0	0	0
Mouse	+ or 0	0	0	0

Data from Dawson, Hobby, and Olmstead (1938, Table on p. 142).

Modifications of Virulence in Vitro and in Vivo.—The conditions which govern the change of a culture from one dissociative phase to another *in vitro* are poorly understood. In general, the smooth and mucoid forms undergo variation to the avirulent R forms when the culture is allowed to age, when it is grown under unfavorable conditions or in the presence of the specific anti-serum. As we have seen, these changes probably correspond to the selection of avirulent mutant forms normally occurring in

any virulent culture (Chapter V:4). It is very likely that the same process is responsible for the emergence of attenuated variants of the tubercle bacillus, such as the well known BCG form (Calmette, 1920; Frimodt-Møller, 1939; Kiorhoe, 1941; Petroff and Steenken, 1930). The virulence of bovine tubercle bacilli can be rapidly decreased by growing the culture in media at pH 6.0 to 6.4. Under these conditions, R colonies soon outnumber the S colonies which are best maintained in media at pH 6.8—a reaction also most favorable for the maintenance of virulence. It must be emphasized that, in the case of the tubercle bacillus as with other bacterial species, the S character of the culture is not always correlated with virulence and that none of the colonial variants derived from an avirulent strain exhibit any significant pathogenicity (Smithburn, 1937) (fig. 29).

Even less is known of the factors which favor the passage from one dissociative form to another *in vivo*. It has been observed that, in chimpanzees, a transformation takes place from the serologically non-type-specific R form of *Hemophilus influenzae* to the serologically specific S form under the influence of infection with the virus of the common cold (Dochez, Mills, and Kneeland, 1932). It is possible also that the presence of specific antibody as a result of infection or of immune therapy, may cause the S \rightarrow R variation to occur *in vivo* as it does *in vitro*.

In any event, it is important to remember that decrease or loss of virulence can occur without the loss of the specific antigenic component which characterizes the smooth form (Eaton, 1934; Schiemann, 1929; Wilson, 1928, 1930). Conversely, it is often possible to increase the virulence of a smooth form by a number of techniques, as by animal passage, without this change being accompanied by any detectable modification of the antigenic structure (Hadley and Wetzel, 1943). The S character of a culture is therefore only one of many factors concerned in virulence; it is a necessary but not a sufficient determinant of it. Thus, there are many strains of salmonella and other Gram-negative organisms which possess Vi antigens characteristic of the virulent form of the typhoid bacillus, but which exhibit no

pathogenicity (Almon, 1943; Bornstein, 1943; Kauffmann, 1941b; Luippold, 1942, 1944; Wilson, 1928). Similarly, many strains of

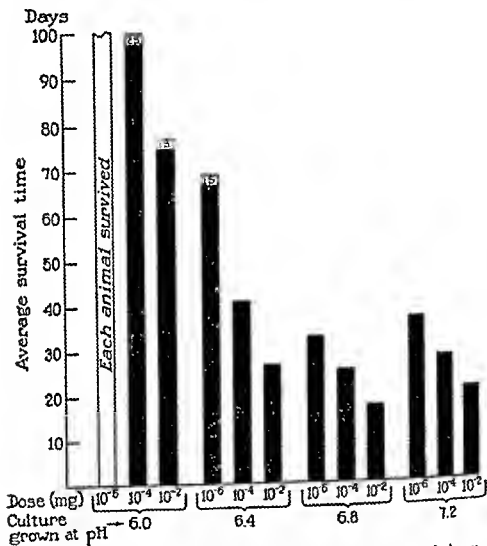


FIG. 29.—Average survival time of twelve groups of four animals inoculated intracerebrally with various doses of tubercle bacilli grown at different pH values. S preceded by a figure in the unshaded portion of a column indicates the number of survivors in that group. (From Smithburn, 1937, chart 1, p. 651.)

group A streptococci fail to cause infection of mice or rabbits even though they produce the M protein (Todd and Lancefield, 1928). The other properties which are required to establish an infection are often referred to as invasiveness and communicability, words

which, as we shall see later, may cover a great many different attributes (Coburn and Pauli, 1941; Menkin, 1936).

Resistance of Bacteria to the Bactericidal Effect Induced by Complement.—The bacteriolytic and bactericidal effects which require the participation of antibody and complement were first described in the case of the cholera vibrios, and have since been shown to affect a great many Gram-negative species. The Gram-positive organisms are, on the contrary, resistant to this action. Ignorance of the mode of action of complement precludes for the time being any hypothesis concerning the contrasting behavior of the two groups of bacteria in this respect. In general, the R nonspecific variants of bacteria are much more susceptible to immune bacteriolysis than the corresponding S and mucoid forms, and we have already mentioned that the specific antigens (the O polysaccharide antigens in particular) possess the remarkable property of inhibiting the effects induced by the antibody complement system. (Cundiff and Morgan, 1941; Thibault, 1939; Thjøtta, 1920) (Tables 25, 26).

Although so much emphasis has been placed on the role of the specific polysaccharide antigens as inhibitors of immune bacteriolysis, it must be pointed out that other antigens can also be concerned in this phenomenon. Thus, immunization of experimental animals with an extract of an R nonspecific variant of *Shigella dysenteriae* elicits the production of antibodies which, in association with complement, can lyse not only R Shiga bacilli, but also the specific S form of the same species and even S forms of Flexner and Sonne strains of dysentery bacilli. It appears certain, therefore, that there exist components of the bacterial cell common to the R and S variants of different shigella strains, which play a part in immune bacteriolysis (Dubos *et al.*, 1944).

The greater resistance of the S forms to the bactericidal effect of normal serum may contribute to the fact that they can survive and multiply in the animal body more readily than the R forms; the following example illustrates this view. Certain smooth strains of *Shigella dysenteriae* are capable of establishing a fatal

infection in mice following the intracerebral injection of small amounts of culture (LD_{50} 0.00005 cc.). There has been obtained from one of these strains an R variant which still possesses the toxigenic properties of the parent strain, but which has lost the ability to produce the O antigen and most of its virulence for mice (LD_{50} 0.02 cc.; R cultures were recovered at autopsy). Study of comparative susceptibilities of the two variant forms to the bactericidal action of normal mouse serum reveals that it takes ten to one hundred times as much serum to kill the same number of S forms as is required for the R variants. This example provides, therefore, a striking correlation between ability to establish an infection and susceptibility to the bactericidal effect of the serum of the normal host (Dubos, *et al.*, 1944).

Resistance of Bacteria to Phagocytosis.—Bacteria in the specific dissociation phase (mucoid, smooth, matt character of the colony) are in general more resistant to phagocytosis than the R nonspecific variants of the same species. The fact that antibodies directed against the antigens characteristic of the specific phase (the O and Vi antigens of Gram-negative bacilli, the capsular polysaccharides of pneumococci, the M proteins of group A hemolytic streptococci, etc.) are capable of stimulating phagocytosis of the homologous bacterial type by normal leucocytes, constitutes suggestive evidence for the view that these specific antigens account for the resistance of the cells which possess them, and thus contribute to the property of virulence. However, as in the case of resistance to immune bacteriolysis, it is likely that components of the bacterial cell other than the type specific antigens, can interfere with the phagocytic reactions. Thus it will be shown in Chapter VII:2 that preparations of the somatic nonspecific carbohydrate (C) of pneumococci inhibit the phagocytosis of these organisms in a serum leucocyte mixture (Enders, Wu, and Shaffer, 1936). There is, unfortunately, too little information available to evaluate the comparative importance of the different bacterial products in the phagocytic reaction. The fact that much of the following discussion is devoted to the relation between encapsulation and phagocytosis should not convey the impression

that the two phenomena bear an exclusive relationship one to the other.

Capsule and Phagocytosis.—The relation of the bacterial capsule to phagocytosis and virulence was recognized early in the case of the streptococcus, of the anthrax bacillus, and of the pneumococcus (Bordet, 1897; Preisz, 1911; Stryker, 1916). It is with the latter bacterial species that there has been obtained the most accurate information concerning, not only the nature of the capsular material, but also its physiological properties (Avery, 1932, 1933). It will be recalled that the capsular materials of pneumococci consist essentially, if not exclusively, of polysaccharides, the composition of which varies from one type to another. It is also known that virulent pneumococci, isolated from pathological material, are always in the encapsulated form, whereas the noncapsulated variants do not occur in disease and are never virulent for experimental animals. The relation of the pneumococcus capsule to virulence is confirmed by much additional evidence. (a) Immunization of mice and man with one of the purified capsular polysaccharides leads to the production of antibodies which protect against infection with pneumococci of the homologous type, but which are ineffective against infection with organisms of heterologous types (Francis and Tillett, 1930; Schiemann and Casper, 1927). (b) Immunization of rabbits with the capsular polysaccharide of type III pneumococcus coupled to horse globulin results in the production of antisera capable of conferring specific protection against infection with this pneumococcus type (Avery and Goebel, 1931; Goebel and Avery, 1931). (c) Enzymes which specifically hydrolyze the capsular polysaccharides destroy the capsule, both *in vitro* and *in vivo*, destroy at the same time the serological specificity of the polysaccharides, and protect experimental animals against infection (Chapters II:4 and IV:3) (Avery and Dubos, 1931; Dubos and Avery, 1931; Francis, Terrell, Dubos, and Avery, 1934; Goodner, Dubos, and Avery, 1932; Shaw, 1937; Sickles, 1940; Sickles and Shaw, 1933, 1935, 1941).

The effect of the capsule upon the course of the infectious

swelling of the capsule which has been described under the name of the "Quellung" reaction (Etinger-Tulczynska, 1933; Neufeld and Etinger-Tulczynska, 1931) (fig. 16), and it is this reaction which apparently so modifies the properties of the polysaccharide that the latter no longer interferes with phagocytosis.

Enzymatic Destruction of the Capsules of Pneumococci.—The antiphagocytic action of the capsule is demonstrated strikingly by the use of enzymes which hydrolyze the capsular polysaccharide. Pneumococci of type III injected intraperitoneally into normal mice multiply rapidly in the peritoneal exudate and are surrounded by well developed capsules. No phagocytosis is observed, and, in fact, leucocytes appear to be maintained at a definite distance from the infective agents by a sort of negative chemotactic effect. When the infected animals are treated with a preparation of an enzyme capable of hydrolyzing the type III capsular polysaccharide, the capsules are rapidly destroyed *in vivo* and are no longer detectable when stained preparations of

FIG. 31.—(See opposite page.) 1. Photomicrograph of a stained preparation of the peritoneal exudate of a mouse 2 hours after intraperitoneal injection of 0.01 cc. of a virulent culture of Type III pneumococcus. The bacteria show well-defined capsules and no evidence of phagocytosis is seen. Many polymorphonuclear and a moderate number of mononuclear leucocytes are present. Gram stain, $\times 1000$. 2. Photomicrograph of a corresponding preparation of the exudate of a mouse 2 hours after receiving the same amount of culture together with 0.5 cc. of a preparation of the specific enzyme. The bacteria are devoid of capsules. Polymorphonuclear leucocytes predominate and phagocytosis is evident. Gram stain, $\times 1000$. 3. Photomicrograph of a stained film of the peritoneal exudate of a mouse 4 hours after injection with 0.01 cc. of culture alone. The bacteria are increased in number, encapsulated, and extracellular. The cellular elements are polymorphonuclear and mononuclear leucocytes in about equal numbers. Gram stain, $\times 1000$. 4. Photomicrograph of a corresponding preparation of the exudate of a mouse 4 hours after receiving the same amount of culture together with 0.5 cc. of a preparation of the specific enzyme. Marked phagocytosis has occurred and only an occasional organism is seen outside the accumulated leucocytes, nearly all of which are of the polymorphonuclear type. Gram stain, $\times 1000$. Differences in the density of the backgrounds of the four figures are due to the use of color screens in the photographic reproductions. This technique, however, alters none of the essential details observed in the original microscopic preparations. (From Avery and Dubos, 1931, plate 4, p. 89.)

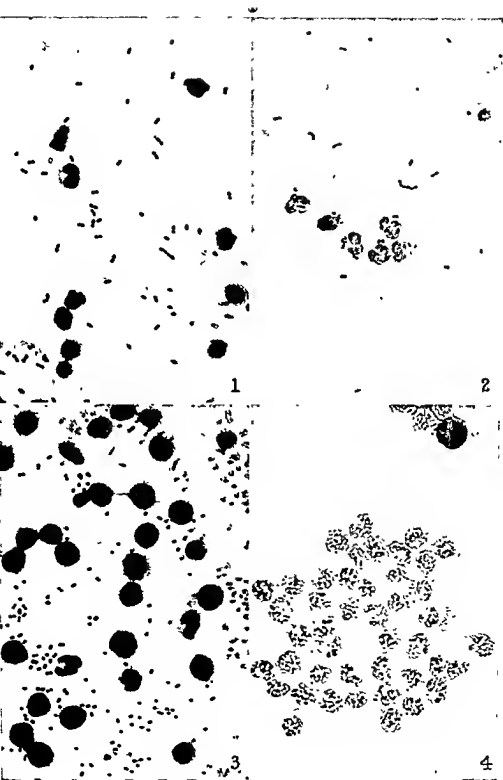
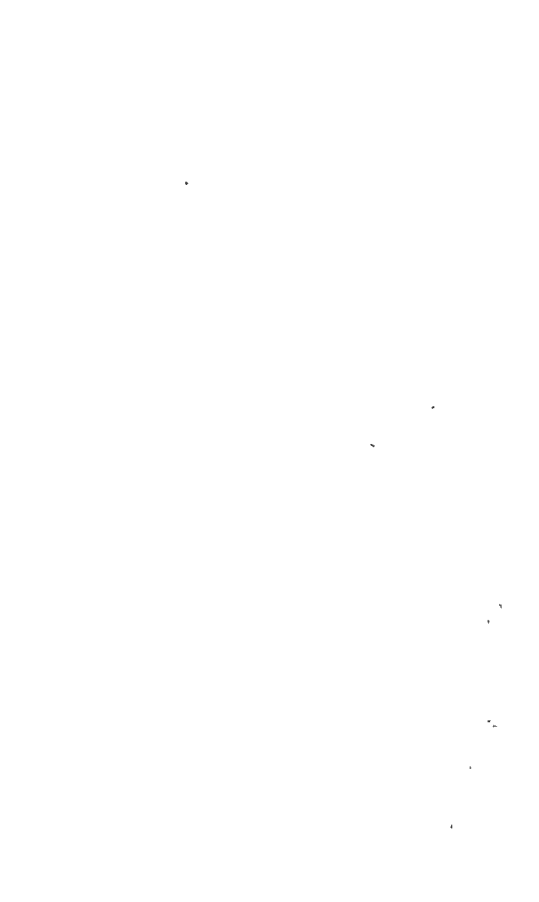


FIG 31



the peritoneal exudate are made one hour after treatment. Phagocytosis begins immediately and after a few hours the peritoneal exudate appears free of pneumococci except for those that are undergoing degeneration within the leucocytes. It must be recalled that, *in vitro*, the enzyme does not affect, either the viability of pneumococci, or their ability to produce the capsular polysaccharide, since, immediately upon their transfer to medium free of the enzyme, they grow again in the encapsulated form. The action of the enzyme is limited to the destruction of the polysaccharide itself, a fact which demonstrates that the inhibition of phagocytosis is due to the activity of this substance (Avery and Duhos, 1931) (Chapter II:4) (fig. 31).

Antiphagocytic Effect of the Free Capsular Substance.—All the facts presented so far are compatible with the view that the polysaccharide exerts its antiphagocytic effect when it is organized as a capsule around the bacterial cell. There are a few observations, however, which indicate that the free soluble polysaccharide may exhibit definite physiological activities, above and beyond the role which it plays as a mechanical protective envelope, as a barrier between the somatic portion of the bacterium and the forces which tend to destroy it. It has long been known that extracts or autolysates of virulent pneumococci have the power of inhibiting the phagocytosis of avirulent pneumococci (Rosenow, 1907), and more recent studies indicate that the specific carbohydrates may be responsible for this anti-opsonic effect. As stated earlier, avirulent pneumococci are phagocytized by normal leucocytes resuspended in normal serum, whereas they grow readily in the serum leucocyte mixture when minute amounts of solutions of the purified capsular polysaccharides are added to the systems. It appears, therefore, that the specific polysaccharides need not be present around the cell in the form of a capsule to be able to inhibit phagocytosis, but that on the contrary they retain this activity in solution (Sia, 1926). The fact that, by injecting into mice type II polysaccharide together with relatively avirulent pneumococci of the same type, it is possible to induce a fatal infection which could not be established by the

pneumococci alone, indicates that *in vivo* as well as *in vitro*, the capsular substance facilitates the infectious process even when present in solution, as well as in the form of a capsule, probably by inhibiting phagocytosis (Felton and Bailey, 1926). The inhibitory effect of extracts from pneumococcic lungs on the bactericidal action exerted by whole blood appears greater than that of the purified polysaccharides, an observation which suggests that these substances may exist in a physiologically more active form during the infectious process (Ward, 1932).

Correlation between Encapsulation and Virulence in Different Microbial Species.—Although capsule formation has not been demonstrated in all pathogenic bacteria, there are many examples other than the pneumococcus, in which interesting observations have been made concerning the relation of capsule to virulence. Thus, phagocytes are not capable of ingesting anthrax bacteria after these organisms have developed capsules in the animal body, although phagocytosis readily occurs when the bacteria are introduced in a nonencapsulated state, as when grown in ordinary broth culture (Grüber and Futaki, 1907). The capsule of *B. anthracis* consists of a polypeptide made up of d-glutamic acid. Little is known of the physiological activity of this substance, and of the role that it plays in infection (Ivánovics, 1938b, 1939). An encapsulated strain of *Staphylococcus aureus*, highly virulent for guinea pigs, was found to lose its virulence when the capsule was lost as a result of dissociation; encapsulation and virulence could be restored by guinea pig passage (Gilbert, 1931).

Many strains of streptococci also occur in the encapsulated form in pathological material (Dawson, Hobby, and Olmstead, 1938; Hobby and Dawson, 1937; Seastone, 1934, 1939). In this case, the capsular material appears to be identical with hyaluronic acid, a constituent of normal animal tissues (Kendall, Heidelberg, and Dawson, 1937). There is some evidence that it plays a part in the property of virulence and that the greater resistance of young cultures of hemolytic streptococci to phagocytosis is due to the larger amount of capsular material which surrounds the young cells (Hare, 1929, 1931; Seastone, 1934; Ward and Lyons

1935). Moreover, the repeated injection of different preparations of hyaluronidase, an enzyme which destroys this capsular material, affords some protection to mice and guinea pigs infected with virulent streptococci (Blundell, 1942; Hirst, 1941; McClean, 1942; Seastone, 1943).

Among pathogenic Gram-negative bacilli, the presence of a capsule and its role in virulence has been established for the pneumobacillus of Friedländer (Julianelle, 1926; Kuryłowicz, Włodzimierz, and Mikulaszek, 1937; Randall, 1939); *H. influenzae* (Pittman, 1931); *H. pertussis* (Shibley and Hoelscher, 1934); the brucella (Huddleson, 1941); *Pasteurella pestis* (Rowland, 1914; Schutze, 1939) etc. There have also been reports of encapsulated typhoid bacilli, although there is no convincing evidence that, in this case, the capsule is of importance in virulence (Morgan and Beckwith, 1939). The Gram-negative bacilli, which are susceptible to the immune lysis mediated by complement, offer an opportunity for the demonstration of the fact that this type of bacteriolysis is prevented by the presence of a capsule (Danysz, 1900). This phenomenon is illustrated in the case of influenza bacilli, the encapsulated virulent strains of these organisms are very resistant to the lytic action of sera which are active against nonencapsulated forms (Wright and Ward, 1932).

There is no doubt, therefore, that the possession of a capsule often increases the resistance of the bacterial cell to the destructive action of phagocytosis and of the bacteriolytic antibodies, and thus contributes to virulence. On the other hand, there are several highly pathogenic species for which no true capsule has been demonstrated as yet. We may point out at this time, however, that according to a view developed earlier, the capsule is not a true morphological structure, but corresponds to the accumulation of viscous material around the cell (Chapter II:4). Since the capsular material can, in some cases at least, exert its physiological effect when free in solution as well as when present around the cell, it remains possible that many non-encapsulated species produce similar substances which, because of higher diffusibility, do not accumulate as a capsule demonstrable by stain-

ing reactions, but which exert a physiological effect just the same. This view is compatible with the observation that noncapsulated bacteria at times show swelling or enlargement under conditions in which their offensive activities come into play, a change which has been claimed to be due to alterations in the peripheral portion of the bacterial cell (Eisenberg, 1908). Such forms have been spoken of as "thierische Bazillen" because they occur most frequently after residence in the animal body (Bail, 1911).

Phagocytosis does not necessarily mean the death of the ingested bacteria; in some cases, indeed, the bacteria produce substances, the leucocidins, which destroy the leucocytes which have engulfed them. In other cases, the ingested bacteria are protected against inimical agencies by the phagocytic cells (Fothergill, Chandler, and Dingle, 1937; Lyons, 1937; Lurie, 1942; Rous and Jones, 1916). It has been shown, however, that whereas phagocytized staphylococci and tubercle bacilli can survive within the leucocytic cells of the normal animal, they die rapidly when the animal has been immunized against the homologous organism (Lyons, 1937; Lurie, 1942) (Chapter VII:3).

Finally, it must be emphasized again that encapsulation is not synonymous with virulence. There are many strains of pneumococci, streptococci, influenza bacilli, etc., which although possessing well developed capsules and belonging to serological types among which are known to occur extremely virulent strains, are completely avirulent for experimental animals. Virulence involves, as we have seen, the possession by the bacteria of many different attributes, of which capsule production is only one.

3. FACTORS AFFECTING THE INVASIVENESS OF THE PARASITE

Adaptation of the Parasite to Multiplication in the Physico-chemical Environment of the Animal Body.—After considering the ability of the parasite to resist the destructive forces of the normal host, it is now necessary to discuss the power of the parasite to multiply in the animal body. The influence of the environment offered by the tissues of the host upon the multiplication

of the parasite was first illustrated by the demonstration that the chicken, normally refractory to anthrax, becomes susceptible after its temperature has been lowered by immersion in cold water (Pasteur, Joubert, and Chamberland, 1878). It is obvious that this experiment is not as conclusive as its simplicity would indicate, since, in addition to lowering body temperature, immersion in cold water results in a number of modifications in the physiology and in the normal defense mechanism of the bird which may greatly affect the infectious process.

There are several other examples of increase in susceptibility to a given pathogen following modification of the temperature of the host. Thus it has been possible to infect frogs with anthrax bacilli and lizards with plague bacilli by keeping these animals at 35° C. and 26° C. respectively (Bouley and Gibier, 1882; Gibier, 1882). Furthermore, there are many interesting correlations between the optimal growth temperature of a parasite and the normal temperature of the tissues of the host for which it is pathogenic. Most cultures of type III pneumococcus are not virulent for rabbits and the strains which are capable of producing a fatal disease into these animals also appear capable of surviving and multiplying at 41° C., a temperature rapidly attained or exceeded by the body of the rabbit during infection (Enders and Shaffer, 1936; Rich and McKee, 1936). Cells of *Treponema pallidum* localized in the inguinal and popliteal lymph glands of rabbits and guinea pigs inoculated with syphilitic material, and in the spleen and brains of infected mice, are more resistant to heat than those occurring in dermal lesions and are at the same time more pathogenic (Bessemans, Van Haelst, and De Wilde, 1935). Similarly, the treponema present in human lymph glands are not destroyed by a temperature at 42° C. for one to three hours, whereas those found in skin lesions are killed when maintained at 41° C. for two hours (Neymann, Lawless, and Osborne, 1936). Finally, there exists a striking correlation between the optimum temperature of the human, bovine, avian, and reptile strains of the tubercle bacillus and the normal body temperature of their animal hosts (Seitz, 1929).

It is evident, therefore, that strains of pathogenic microorganisms differ in respect to their optimal temperature for survival and multiplication, and that this difference can be of some significance in affecting their pathogenicity for a given host. Furthermore, one can often modify the optimal temperature of a given strain by a process of training *in vitro*, a phenomenon which, as we have seen, corresponds to the selection of those forms within any normal population which are adapted to survival and growth at higher or lower temperature. It is scarcely to be doubted that a similar selection can occur *in vivo* and that it is of some significance in the adaptation of a given organism to parasitic life within a given host.

There are, naturally, a great variety of other factors such as specific nutrients, gaseous environment, acid base properties, etc., which affect the ability of a microorganism to multiply in the animal body. Although they do not offer convincing evidence of their relation to virulence, the following examples will serve to illustrate the nature of the problem. Most, if not all, microorganisms utilize CO_2 in their metabolism, and exhibit a sharp optimum with reference to the CO_2 tension at which they grow best. In the case of a certain strain of the tubercle bacillus, this optimum was found at 2.5% CO_2 at pH 7.4. Pneumothorax therapy increases the CO_2 tension in the collapsed lung, and it has been suggested that this unfavorable gaseous atmosphere may be effective in reducing the growth of the parasite (Kirchner, 1930; Davies, 1940).

Encapsulated pneumococci can give rise to many types of variants independently of the classical $\text{M} \rightleftharpoons \text{S} \rightleftharpoons \text{R}$ variation. Some encapsulated variants which give only phantom colonies at 37° C. have been termed the P-C forms. They undergo rapid autolysis at 37° C., but this autolytic process is inhibited by high CO_2 tension, lower oxygen tension, and more strongly reducing and less alkaline conditions than those which usually prevail in ordinary culture media. Interestingly enough the P-C variants have been frequently isolated from blood, sputa, and the

lungs of pneumonia patients, a fact which is not surprising since conditions in the body are nearly optimal for their growth (Eaton, 1934; 1935). Whether these variant forms are the ones which are predominant in the lesions caused by pneumococci, whether, for instance, they represent the essential infective agent in lobar pneumonia, has unfortunately not been established. Certain strains of group A streptococci also appear to undergo a similar variation without any modification of their type specificity; they give rise to atypical forms the growth requirements of which (pH, CO₂ and oxygen tension) are similar to the conditions prevailing in the human throat. These variant forms seem to be endowed with greater invasiveness and to be more capable of initiating epidemics (Coburn and Pauli, 1941).

The physiological state of the culture at the time of infection is one of the other factors which affects the adaptation of the parasite to multiplication in the *in vivo* environment. The bacterial cell undergoes, during the growth process, a cytomorphosis which expresses itself not only by modifications of cellular morphology, but also by profound alterations of physiological and biochemical properties (Chapter V:2). It is not surprising, therefore, that the minimal number of bacteria required to establish a fatal infection varies greatly according to the age and vegetative activity of the culture. Thus, with a certain strain of streptococcus, it was found that the minimal lethal dose (for the mouse) of the 5, 24, and 72 hour cultures were, respectively, 1,700,000, 26,000,000, and 102,000,000 viable cells; transfer from the 3 day old culture yielded a new culture which, after 5 hours' incubation, had an MLD corresponding to 540,000 organisms. It is clear, therefore, that older cultures are much less invasive than cultures in the stage of active multiplication. This change in virulence must be differentiated from the alteration caused by bacterial dissociation or animal passage, since it is very transitory and since full virulence is restored merely by making a new transfer of the culture to obtain young cells in the active vegetative state (Felty and Bloomfield, 1924). Similar modi-

increasing the permeability of connective tissue (McClean, 1941; Duran-Reynals, 1942). Thus staphylococcus strains can be grouped according to the extent of the lesions which they induce when injected intradermally into rabbits, and this in turn is an expression of the amount of spreading factor which they secrete (Duran-Reynals, 1933). Active substances of the coagulase, fibrinolysin, and spreading factor types are produced by most pathogenic bacteria, and these different bacterial constituents and products are important in determining the histological reactions associated with different types of infection (Goodner, 1931, 1933). Each bacterial strain is characterized not only by the possession of one or several of these agents, but also by the fact that each type of activity may exhibit some degree of host specificity, a given strain of streptococcus being, for instance, lytic for human fibrin, but not for rabbit or fowl fibrin (Reimer, 1936). Like virulence, invasiveness is only a word summarizing the immense variety of factors which condition the ability of a given parasite to multiply in a given host.

4. TOXIC ACTION OF BACTERIA

Causes of Toxemia.—There is no apparent reason why the mere presence in the tissues or body fluids of any reasonable number of bacterial cells should exert any harmful effect on the host that harbors them. No mechanical theory of the pathogenic action of bacteria is compatible with our knowledge of the way in which the tissues deal with inert particles which have gained access to them; the basis of the harmful effects of most bacterial infection is certainly chemical. It is through a disturbance of the normal physiological processes of the host that pathogenic bacteria cause those symptomatic and pathological manifestations which characterize each individual infectious disease. In some cases, the infective agent interferes with the normal physiology of the host by competing with the latter for some factor essential to vital processes; in other cases, the parasite may release simple metabolic products which, without being essentially toxic, alter the

course of the biochemical events of normal tissue metabolism. These possibilities have been so little explored that they can only be stated in general terms.

In most cases, however, it has been established that pathogenic bacteria produce a variety of substances endowed with great pharmacological activity and which are undoubtedly responsible for many of the clinical and pathological manifestations of each infectious agent. These substances are referred to as bacterial toxins or bacterial poisons; they have often been classified into exotoxins and endotoxins, the former being readily released into the medium in a soluble form free of the cells, whereas the latter characteristically remain bound to the cellular structure. The typical exotoxins, diphtheria, or tetanus toxin, for instance, exhibit characteristic pharmacological activity, are fatal in very small dose, are thermostable and readily elicit the production of antitoxins which neutralize them specifically and in accordance with the law of multiple proportions. The typical endotoxins, on the other hand, such as cholera or typhoid toxin, produce nonspecific lesions and symptoms in experimental animals, are much less active in terms of minimum fatal doses, are relatively stable to heat, and in general do not stimulate the production of effective antitoxic sera (Zinsser, Enders, and Fothergill, 1939, page 78). There are, however, so many exceptions to these general statements that the validity of the classification can be questioned. It is very likely, in particular, that many of the classical exotoxins are not secretion products, but are released in solution only as a result of autolytic breakdown of the cells which produce them. For instance, diphtheria and tetanus toxin as well as the exoneurotoxin of *Shigella dysenteriae* reach high concentration in the liquid medium only when most of the bacteria are dead or dying; this fact has been particularly well established in the case of the Shiga bacillus (Okell and Blake, 1930).

It is probable that, to exert their full poisonous action, the toxic components of the bacterial cell must be released in solution in the animal body. This view would explain the observation that guinea pigs immunized against cholera die more rapidly than con-

trol animals when injected with large numbers of these microbial agents, since it is known that the vibrios rapidly undergo lysis in the body of the immunized animals (Pfeiffer, 1894; Pfeiffer and Issaëff, 1894; Pfeiffer and Wassermann, 1893). In fact, it has even been claimed that many so called toxic proteins of bacterial or other origin are not primarily toxic, but become poisonous only after being acted upon by the enzymes of the host; there is, however, no convincing evidence to support this view (Vaughan and Novy, 1902; Vaughan, Vaughan, and Vaughan, 1913).

Factors Affecting the Production of Bacterial Toxins.—As in the case of other bacterial activities, toxin production within one species varies greatly from strain to strain, from one variant form to another within the same strain, and also depending upon the environmental conditions under which the organism is growing. The fact that different strains of the same species vary greatly in their toxigenicity is of obvious importance in conditioning the severity of the disease which they cause, and also for the *in vitro* production of toxin and toxoid (Mueller, 1941a, b; Zinnemann, 1943). Although the toxigenicity of many bacterial strains has remained constant in the laboratory for a considerable number of years, and has thus permitted the development of highly standardized methods for the production of toxin and toxoid, there are well authenticated cases of variation of this property.

Toxigenicity can vary independently of the other properties of the culture involved, of its antigenic constitution in particular. Thus, the production of staphylococcus toxin, streptolysin, Welch toxin, Shiga neurotoxin, etc., is independent of the state of dissociation (M, S, R) of the culture (Haherman, 1941; McGaughey, 1933; Steahhen, 1943; Todd, 1942).

It is only in the case of the Gram-negative bacilli that evidence has been offered that the smooth forms possess a toxin, the so called "complete antigen," which is not present in the rough forms. The S forms of these organisms are often more toxic than the R variants, and much of the toxicity of the former follows the phospholipid-peptide-polysaccharide complex which determines the specific antigenicity of the cell (Chapter IV:2). It must

he kept in mind, however, that there are R variants of the *Bacteriaceae* which are essentially as toxic as the S forms, and that the nature of the toxic reactions induced are apparently essentially the same in the two cases. Much indeed remains to be done before the nature and distribution of toxic material in the Gram-negative bacteria is completely clarified.

The influence of environmental factors on toxin production deserves special mention. The effect of the gaseous environment is illustrated by the fact that staphylotoxin is produced in good yields only when the staphylococcus culture is agitated with a gas mixture containing approximately 10 per cent CO_2 (Burnet, 1929b, 1930a, 1931); the so called exoneurotoxin of Shiga is produced only when the cells grow by aerobic metabolism (Dubos, Hoberman, and Pierce, 1942; Olitsky and Kligler, 1920; Waaler, 1936). Although production of diphtheria toxin is always carried out by growing the organism in shallow layers and by favoring pellicle formation during growth, it is not known whether this technique only increases growth with a proportional increase in yield of toxin, or whether it has a specific effect on toxin production. It appears that not only the availability of oxygen, but also CO_2 tension may affect both phenomena (Plastridge and Rettger, 1929). The effect of the concentration of inorganic iron in the medium on the yield of diphtheria toxin supplies striking evidence that the synthesis of bacterial protoplasm is not necessarily related to the yield of toxin (Mueller, 1941a; Mueller and Miller, 1941; Pappenheimer and Johnson, 1936). The optimal amount of iron varies greatly with the composition of the medium and with the strain. In general, however, it appears that the growth does not occur in the absence of the metal and that consequently the amount of growth and, parallel with it, the amount of toxin, increase up to an optimum level. Increase in growth is still observed beyond that point, but toxin production decreases rapidly as the concentration of inorganic iron increases (Table 30). The importance of these facts for the practical matters of toxin and toxoid production is obvious, and it is also probable that they are of significance in conditioning the toxemia of diphtheria

TABLE 30

THE INFLUENCE OF IRON ON THE PRODUCTION OF DIPHTHERIA TOXIN

Flask	$\mu\text{g Fe/ml added}$	Lf/ml	MLD/ml
1	none	17.5	700*
2	0.04	20	800
3	0.2	17.5	700*
4	0.4	10	400*
5	0.8		60
6	2.0		10
7	4.0		3

* Calculated on the basis of 40 MLD/Lf, which was determined experimentally on the toxin from flask No. 2.

Data from Mueller (1941, Table 1, p. 347).

infections (Mueller, 1941b; Zinnemann, 1943). The concentration of iron and other elements in the medium is also important for the production of tetanus toxin (Mueller and Miller, 1943) and of Shiga neurotoxin (Dubos *et al.*, 1944), etc. Furthermore, many other observations illustrate the fact that bacterial growth and production of toxin are not necessarily related. Thus it is possible to obtain abundant growth of *Bacillus welchii* without any production of α toxin (lecithinase) and there is indication that a certain substance, of unknown nature but abundantly present in casein and muscle, is necessary for production of the lecithinase although growth proceeds normally in its absence.

The Nature of Bacterial Toxins.—So few of the bacterial toxins have been obtained in a reasonable state of purity that knowledge concerning their chemical nature must necessarily rest on circumstantial evidence. In fact, it is only in the case of the diphtheria toxin that adequate criteria of purity and accurate knowledge of composition have been published. Constancy of analytical composition and of biological activity of preparations obtained by different methods and evidence of homogeneity obtained by solubility measurements, sedimentation, and migration

in an electric field have permitted the identification of diphtheria toxin with a well defined protein molecule, the composition and properties of which are summarized in Table 31 (Eaton, 1938; Pappenheimer, 1942).

TABLE 31

COMPOSITION AND PROPERTIES OF DIPHTHERIA TOXIN

Carbon	51.47%
Hydrogen	6.75
Nitrogen *	16.0
Sulfur	0.75
Phosphorus	<0.05
Ash	1.4
Amino nitrogen	0.98
Tyrosine	9.5
Tryptophane	1.4
Arginine	3.8
Histidine	2.4
Lysine	5.3
Specific rotation	- 39°
pH of isoelectric point †	4.1
Molecular weight	72,000.
Svedberg Dissymmetry No., <i>f</i> / <i>f</i> ₀	1.22
Ratio of major to minor axes	4.7
Nitrogen, specifically precipitable by antibody	95-98%
MLD per mg.	14,000.

* Corrected for ash content.

† Cataphoresis.

Data from Pappenheimer (1942, Tables 1 and 4, pp. 277 and 282).

It may be appropriate to mention at this time the tuberculo-protein concerned in the tuberculin reaction. Although tuberculin exhibits little primary toxicity for cells or animals, it may be more toxic in the native form in which it exists in the tubercle bacillus. In any event, tuberculo-protein gives rise to sensitization phenomena which express themselves by toxic reactions recognized not only *in vivo*, but also by tissue culture methods *in vitro*

(Moen, 1936; Moen and Swift, 1936; Rich and Lewis, 1932). Preparations of tuberculin have yielded a protein of 16,000 to 32,000 molecular weight, which exhibits homogeneity in centrifugal and electric fields and which determines in sensitized animals a tuberculin reaction. This protein, however, does not seem to possess all the sensitizing power of the original culture, and may correspond to the breakdown product of a more complex mother substance present in the tubercle bacillus. Other more antigenic tuberculo-proteins present in preparations of tuberculin have also been studied, but less adequately separated and described (Seibert, 1941; Seibert and Glenn, 1941).

The hemolysins of pneumococci and of streptococci have been obtained in the form of proteins which can undergo reversible inactivation as the result of reversible oxidation of some —SH constituent of the molecule (Shwachman, Hellerman, and Cohen, 1934). Although not obtained in a purified form, the α toxin of *Clostridium welchii* has been shown to be an enzyme, a lecithinase, which decomposes lecithin into phosphocholine and a diglyceride (MacFarlane, 1942; MacFarlane and Knight, 1941). As scarlet fever toxin becomes more and more purified, evidence accumulates of its protein nature, in spite of the fact that it is fairly resistant to heat and to proteolytic enzymes (Krejci, Stock, Sanigar, and Kraemer, 1942). A similar situation exists in the case of the neurotoxin of Shiga. There have been claims that this substance is inactivated by trypsin, but several observers have found it resistant to crude trypsin, pepsin, and papain. The substance becomes insoluble and inactive when precipitated with acetone at room temperature or heated for ten minutes at 80° C. at slightly alkaline reactions (it is more heat stable at neutral or slightly acid reactions); it becomes insoluble and inactive when shaken with chloroform, probably as a result of surface denaturation of a protein constituent (Dubos *et al.*, 1944; Haas, 1940).

There are thus a large number of toxic products of bacteria for which there is convincing or suggestive evidence of their protein nature. Since many if not all of these toxins are usually classified among the exotoxins, it is interesting to contrast them with the endotoxins of the *Bacteriaceae* for which there is evi-

dence that the poisonous property accompanies the specific polysaccharide. Although the complete toxic antigens of these organisms probably occur in the cell as phospholipid-protein-polysaccharide complexes (Chapter IV:2), it must be emphasized that there is no accurate information concerning the component parts of these complexes which are responsible for toxicity. Removal of the phospholipid leaves a toxic, antigenic complex; when, however, the polysaccharide and the protein components are obtained in their intact native form, both retain definite toxicity (Morgan and Partridge, 1940, 1941; Perlman, Binkley, and Goebel, 1944). Whether this residual toxicity is due to incomplete separation of the two components, or to the fact that both are contaminated with some other substance which is the toxic material, has not been satisfactorily determined. The fact that the endotoxins of the Gram-negative bacilli exhibit striking similarity in their pharmacological and pathological effects, irrespective of the bacterial species from which they are prepared, indicates the existence of a toxic component common to all the bacteria of this group and serves as another criterion to separate them from the Gram-positive species (Ayo, 1943; Delafield, 1932; Schwartzman, 1937; Zahl, Hutner, and Cooper, 1943b). It is not established, therefore, whether the specific polysaccharides of the *Bacteriaceae* are toxic *per se*, or whether they owe their toxicity to some other substance combined with them. It is well known that in the purified and degraded forms in which they were first studied, the capsular polysaccharides of pneumococci and of the Friedländer bacilli are not toxic, except for their ability to give rise to anaphylactic reaction in sensitized animals. It must be remembered, however, that these substances exist in the cell in a more complex form which may be toxic. Furthermore, toxicity should not be measured only in terms of lethal action, since it may take the form of physiological effects which are not readily detectable by ordinary techniques.

Let us mention, finally, the recent claims concerning the existence in the tubercle bacillus of a toxic substance, which when injected in amounts of 2 μ g into normal guinea pigs, causes a toxic reaction resulting in extensive lung involvement and death.

This substance can be extracted from the bacilli in solution in paraffin oil and is heat stable and precipitable by dioxane. It appears to be a mycolic acid ester of a polysaccharide, and loses its biological activity when these two components of the molecule are separated by acid hydrolysis (Choucroun, 1939, 1940, 1943).

As stated earlier, "Microbes are chiefly dangerous on account of the toxic matters which they produce. . . . Death in infectious diseases is therefore caused by intoxication" (Roux, 1889; Roux and Yersin, 1888, 1889). It would be of the greatest interest, consequently, to understand how toxins exert their physiological and pathological disturbances, since only then will an insight be gained into the intimate mechanism of infectious diseases. Unfortunately our ignorance of these problems is truly appalling. In only one case, that of the α toxin of *Clostridium welchii*, a lecithinase which splits lecithin into a diglyceride and phosphocholine, has the substrate attacked by the poison been identified (Macfarlane, 1942; Macfarlane and Knight, 1941). Nothing is known of the nature of the "biochemical lesion" for which other toxins are responsible, whether they act by destroying a structure or inhibiting essential metabolic functions. Except in the case of hemolysins and leucocidins, we lack even the knowledge of the tissues and cells upon which the primary lesions are exerted. Toxin action has been analyzed and described only in terms of the secondary phenomena resulting in pathological and clinical manifestations. Further consideration of these matters is outside the scope of our discussion, but it is worth emphasizing again that study of the factors affecting the production of toxins both *in vitro* and *in vivo*, and of the mechanism of their action on susceptible cells and biochemical systems, remains one of the most important and rewarding fields of medical bacteriology.

5. INDEPENDENT VARIATION OF THE DIFFERENT COMPONENTS OF VIRULENCE

The term virulence does not refer to one specific attribute of the infective microorganism, but expresses the ability of a given

agent to establish under well defined conditions a pathological state in a given host (Chapter VI:1). Any variation in the host or the parasite will, therefore, affect the manifestations of virulence. Host variations are outside the scope of the present survey. It is necessary to reiterate, on the other hand, that like the other characters of bacteria, the different components of virulence, such as communicability, resistance to the normal and immune defense mechanisms of the host, invasiveness, production of poisonous substances, etc., may vary independently of each other.

It is obviously difficult to demonstrate the persistence of one of the components of virulence in a bacterial cell which has lost one or more of the others, since the establishment of an infection usually requires the participation of all the factors involved in virulence. Thus, an organism may have retained the invasive property, but cannot manifest it if it has lost the ability to resist the phagocytic action of the host. Fortunately, techniques are available which permit the demonstration that, in nonencapsulated avirulent pneumococci, the potential property to invade persists even though it cannot result in the establishment of the pathological state as long as the organism does not produce capsular material. When nonencapsulated (R) avirulent pneumococci derived from an encapsulated S (mucoid) strain of type III organisms virulent for mice, but not for rabbits, are reconverted to the S (mucoid) form by the addition of a vaccine prepared from an S strain virulent for both animal species, the converted strain is virulent only for mice. Conversely, R forms derived from the rabbit virulent strain which are transformed into the S state by means of a vaccine prepared from the rabbit avirulent organisms, are found to be virulent for rabbits. In both cases, therefore, the converted strain retains the pathogenic properties of the parent strain from which it was derived, even though these pathogenic characteristics cannot manifest themselves, on account of lack of capsule, during passage through the R phase. The factors upon which depend the differences in virulence between the two original strains, are expressions of physiological differences which per-

sist, unrecognized, in the R phase (Shaffer, Enders, and Wu, 1936).

The demonstration of this phenomenon becomes even more striking when pneumococci of different virulence are caused to change their type specificity by way of the R intermediates. Two strains of pneumococcus type III, one virulent for the rabbit, the other avirulent, were transformed into pneumococcus type II. The virulence of the newly derived strains of type II corresponded to that of the original type III strain, indicating that the differences in virulence are related to a cellular factor which persists in the R phase and which is not dependent upon the capsule. In other cases, on the contrary, the cellular factor may be present and yet masked because of the nature of the capsule. When an R variant derived from a virulent strain of type II pneumococcus was transformed into types I, III, and XIV, the types I and III were fully virulent, and the type XIV strain was avirulent (MacLeod and McCarty, 1942).

Virulence thus is an immensely complex property, the summation of many complementary attributes. The infectious agent must be able to penetrate the protective barriers which shelter the host from the environment, it must be able to survive the many defense mechanisms, cellular and humoral, which attack it as soon as it reaches the tissues; it must find an environment favorable for multiplication; and finally it must be able to produce disease, *i.e.*, to produce substances or conditions which cause physiological and pathological disturbances. The most striking property of the bacterial cell is the readiness with which each one of its attributes can undergo variation, independently of the others. Loss by variation of any one of the components of virulence results in loss of ability to establish a pathological state. We need not wonder, therefore, that it is so often difficult to maintain a culture in a virulent state outside the body of the susceptible host, and that, of the countless strains of bacteria which exist in nature, so few are endowed with pathogenicity.

VII

IMMUNIZATION AGAINST BACTERIAL INFECTIONS

Antitoxins and antibacterial substances are, so to speak, charmed bullets which strike only those objects for whose destruction they have been produced.

PAUL EHRLICH

1. TRENDS IN THE DEVELOPMENT OF IMMUNIZING PROCEDURES

E*volution of the Concepts Immunity and Vaccination.*—It has been mentioned that the concept "virulence" underwent a striking evolution as the emphasis in the study of infection shifted from the disease itself to the etiological agent, and finally to the host-parasite relationship (Chapter VI:1). Similarly, the words immunity, immunization, vaccine, and vaccination have suffered considerable distortion of their original meaning during the bacteriological era and these alterations have caused much confusion in the statement of the related phenomena and have perhaps even retarded the growth of immunological philosophy.

Sensu strictu, the word immunity refers to a state of resistance to the effect of a given injurious agent or procedure. In the case of infectious agents, immunity can be brought about by stimulating the production in the host of certain antibodies capable of reacting with the pathogen. On the other hand, antibodies can be produced, not only against bacteria, but also as a specific response to the injection of a great variety of substances into the animal body. Since the laws which govern the production and the manifestations of antibodies are to a large extent independent of the origin and of the nature of the injected material, all related phenomena were grouped in one discipline called immunology.

Antibody production against a given substance or bacterial preparation is commonly referred to as the result of "immunization" with that substance or bacterial preparation, irrespective of whether the eventual outcome is beneficial to the host as in the case of resistance to infection, or unfavorable as in the development of an allergic state. Used in this sense, therefore, the word immunization refers to a technical procedure and no longer to the production of a state of resistance.

The expressions vaccine and vaccination have undergone a parallel evolution. They have their origin in the fact that Jenner produced resistance against smallpox in man by the injection of material obtained from the cow—*vacca*. When Pasteur (1880) observed that birds injected with an old, avirulent culture of chicken cholera became resistant to infection with the virulent form of the same organism, he recognized the analogy of the phenomenon to the relationship between cowpox and smallpox, and felt justified in referring to the attenuated, avirulent bacterial culture as a "vaccine." Subsequently, any bacterial suspension, attenuated or killed, injected into experimental animals or man, has been described as a vaccine, whether it does or does not increase resistance against infection. Indeed, very few vaccines deserve their name, if the word is to be used according to the Jennerian or Pastorian criteria. The production of antibodies, rather than the establishment of a state of resistance, is often the criterion used by experimenter and clinician alike for measuring the result of "immunization" with a bacterial "vaccine."

An attempt will be made in the present chapter to identify these singular components of the bacterial cell—the protective antigens—which induce the production of the protective antibodies upon which depends resistance to infection. Unfortunately, there is at the present time little theoretical basis upon which to determine which cellular structures can elicit a protective reaction. There are, on the other hand, several examples of immunological procedures which undoubtedly give rise to anti-infectious immunity. It may be profitable, therefore, to present a brief survey of the methods of immunization which have proven of practical

value, in the hope of deriving from the results of empirical practices a theoretical basis for future advances.

Immunization with Attenuated Living Cultures.—The fact that recovery from certain contagious diseases is associated with immunity against a second attack of the same infection led very early to the development of empirical practices of immunization. Thus, it has long been known that the inoculation of smallpox material into human beings living under conditions favorable to general good health causes only a mild form of the disease which confers lasting immunity against subsequent exposure. Inoculation of the Aleppo boil to some concealed part of the body was and is still practiced as a preventive treatment against the scars and disfigurement which might follow infection with *Leishmania tropica* (Strong, 1942, p. 305). The first systematic practice of immunization in the western world was introduced by Edward Jenner who established the fact that a mild infection with cowpox protects human beings against the much more severe smallpox (Jenner, 1789). Although cowpox and smallpox are now known to be caused by filterable viruses, they have provided the pattern, and are likely to yield still more pertinent information in the future, for the study of antibacterial immunity. It is generally considered that the viruses of cowpox and smallpox are either derived from a common ancestor, or are at least very closely related. By passage through cattle, cowpox has become so attenuated for man that it incites only a mild and localized form of the disease, which is sufficient, however, to stimulate in the host the production of antibodies capable of protecting against infection, not only with the cowpox but also with the smallpox virus.

It was while working with chicken cholera that Pasteur first perceived the mode of action of the Jennerian method of vaccination against smallpox and recognized that the same principle could serve to establish resistance against bacterial infections. Cultures of chicken cholera which had been allowed to stand without transplantation for a long period of time before being inoculated into normal birds failed to kill them, giving rise in many cases to localized lesions only; moreover, the birds injected with

these old cultures were found to have become resistant to the injection of the fully virulent strains. The old bacterial cultures had, in Pasteur's terminology, become "attenuated" and were analogous to cowpox, attenuated for man by passage through cattle. Just as cowpox constituted a vaccine against the more virulent (for man) smallpox, so attenuated cultures of chicken cholera could serve as a vaccine to protect against infection with the virulent form of the same bacterial species (Pasteur, 1880). These observations have provided a rational philosophy and practical techniques for the study of antibacterial immunity.

Many methods have been advocated for the production of attenuated cultures to be used in prophylactic immunization. Most of them have consisted in growing or maintaining the infectious agent in an unfavorable environment such as: allowing cultures to age under aerobic conditions and in the presence of their own metabolic products; incubating at high temperatures; cultivating in the presence of antiseptics; etc. It is probable that, in reality, the unfavorable environment does not *cause* the bacteria to change from the virulent to the avirulent, attenuated state, but only serves to select these avirulent forms which normally appear as variant, mutant forms in any virulent culture (Chapter V:4, 5). Thus, although the classical BCG strain was obtained by prolonged growth of a bovine tubercle bacillus in the presence of bile, the virulent cultures can be shown to give rise to variant forms which are avirulent on many types of media. Similarly, cultures of the plague bacillus, even though issued from a single cell, often consist of a mixture of fully virulent and attenuated forms which can be separated by colony isolation. Many strains of dysentery bacilli rapidly change from the smooth to the avirulent rough form especially when repeatedly transferred in liquid media. Production of avirulent variants from virulent cultures of many pathogens can also be achieved by cultivating the latter in the presence of specific antiserum; in this case again, it is possible that the antibody acts by favoring a selection of the normally occurring avirulent forms (Chapter V:4).

In any event, the change from the virulent to the avirulent

state is not always complete and is often reversible. The possible persistence of, or the reversal to, virulent forms has led many workers to consider that immunization with living attenuated cultures is a dangerous procedure which, although interesting in theory, should not be used in the case of human beings. Far from being abandoned, however, the method has had several important practical applications. Not only has it been applied on a large scale in the case of certain filterable viruses (vaccinia, yellow fever), but there are also several bacterial infections such as brucellosis and plague in which it has given favorable results on a practical scale.

Immunization with Suspensions of Killed Bacteria.—Analysis of the phenomena associated with recovery from infectious diseases and of those which follow active immunization soon revealed the existence in the serum of the immune host of a variety of antibodies capable of causing agglutination or phagocytosis of the parasitic agents. It was also early recognized that bacteria killed by heat are as readily agglutinated by specific antiserum as the living germs, and that the killed bacteria themselves can also induce in animals the production of specific antibodies (Chapter IV:2). This correlation, and confusion, between the development of resistance to infection and the production of antibodies, led to the belief that protective immunity can be established by the use of killed bacteria as vaccines, and can also be measured by titrating the agglutinins, precipitins, bacteriolysins, bacteriotropins, etc., present in the serum of the immunized host. It is unnecessary to describe the countless types of vaccines and sera which have been advocated for the prophylaxis and therapy of infectious diseases, without adequate appraisal of their effectiveness, let alone of the physiological basis of their mode of action. Immunizing methods of established efficacy have been obtained against very few species of pathogenic bacteria and only scattered facts are available for the formulation of a theory of anti-infectious immunity.

New points of view and new techniques were introduced into the problem of immunization when it was recognized that micro-

organisms are not constant in their structure and behavior, but that on the contrary they can exist in several forms which differ not only in morphology, chemical composition and virulence, but also in their immunizing properties, etc. Just as these discoveries have revealed that the property of virulence is the summation of several independent attributes (Chapter VI), they have led to an analysis of the relative importance of the different antigenic components of the bacterial cell in the phenomenon of resistance, and have helped in the selection of appropriate cultures for the preparation of effective antigens.

Immunization with Extracts or Products of Bacteria.—The fact that a state of resistance can be elicited by the injection of killed bacteria proves that the immune reaction is not directed against some mysterious "living" property of the parasite. Killed bacteria are, so to speak, only a mixture of inorganic and organic substances, and the immune process is directed therefore against one or several of the chemical components of this mixture. In fact, the most active field of immunological research during the past two decades, and one of the most fruitful in discoveries of both theoretical and practical importance, has been the analysis in chemical terms of the antigenic mosaic of the bacterial cell, the separation of its components, the determination of their chemical nature and of their biological properties. It is on the basis of the knowledge thus acquired that an attempt will be made to interpret some of the phenomena of antibacterial immunity.

2. BACTERIAL STRUCTURES CONCERNED IN THE IMMUNITY PROCESS

Relation Between Virulence and Possession of Protective Antigens.—Two independent trends can readily be recognized in the evolution of the methods of antibacterial immunization. On the one hand, efforts have been made to utilize living vaccines consisting of attenuated, avirulent bacterial forms. On the other hand, the possibility of eliciting a state of immunity by the injection of suspensions of killed bacteria or of their products has

permitted the preparation of immunizing agents from highly virulent organisms, recently isolated from pathological material. The two methods obviously differ in their fundamental principle. Indeed, if the killed vaccines owe their immunizing activity to cellular components related to virulence, it is difficult to account for the effectiveness of immunization with avirulent living vaccines. It might be conceived that the living attenuated vaccines are effective by directing the immune reaction against some process essential to the growth or division of bacteria. This explanation appears untenable in view of the fact that, in many cases, the attenuated cultures retain their immunizing properties after the organisms have been killed by heat or other techniques.

The theory that the most virulent forms recently isolated from pathological material are also the best immunizing agents is based on an unjustified generalization from a few experimental facts. It is true that the virulent forms of a number of bacteria have yielded certain antigens such as the capsular polysaccharides of pneumococci, the M proteins of group A streptococci, the O and Vi antigens of the Gram-negative bacilli, etc., which give rise to antibodies of high protective value. It will be recalled, however, that these same antigens, although always present in the virulent forms, occur also in completely avirulent variants (Chapters IV:2 and V). There are, moreover, many examples of cellular components which are characteristic of the virulent strains and which elicit the production of antibodies devoid of any protective power. Finally, certain attenuated strains which have lost the specific characteristic antigens of the virulent forms still possess marked immunizing ability. It is certain, therefore, that the stimulation of protective immunity is not an attribute necessarily correlated with virulence, but is rather dependent upon the presence in the bacterial cell of one or several peculiar structures capable of eliciting the production of certain antibodies which, for reasons not always clear, can interfere with the pathogenic career of the parasite.

The Immunizing Efficacy of Smooth and Rough Variants.—The discovery that, in pathological material, bacteria usually

Goebel, 1925). Other serological relationships between the specific polysaccharides of pneumococci and those obtained from different strains of yeasts and higher fungi have also been found to express themselves in protective immunity (Neill, 1939; Sugg, Richardson, and Neill, 1929).

There are many reports in the literature indicating that, among Gram-negative bacilli, and especially in the salmonella and shigella groups, the S specific variants are generally (but not universally) better immunizing antigens than the R forms (Arkwright, 1927; Greenwood, Topley, and Wilson, 1931; Ibrahim and Schütze, 1928). Since one of the most striking manifestations which accompanies the S \rightarrow R variation in these organisms is the loss of the specific O antigen, it was natural to assume that the specific polysaccharide component of the complete antigen was responsible for the protective immune reaction. Indeed, it will be recalled that the complete antigens of the Gram-negative bacilli can be obtained in solution free of the bacterial cells which produce them, and that they readily give rise to protective antibodies. It is interesting that the pure polysaccharides isolated from the O antigens have not as yet been shown to be antigenic by themselves; they can immunize only when in combination with a special type of protein. Remarkably enough, it is possible to restore specific antigenicity to the nonantigenic, free polysaccharides by recombining them with the protein with which they were associated in the cell or with the homologous protein from other related bacterial species. Thus the free, specific polysaccharide of *Shigella dysenteriae* is not antigenic in mice or rabbits, but elicits the production of antibodies specific for itself after it has been coupled with the proper protein isolated from shigella or eberthella cultures (Partridge and Morgan, 1940) (Chapter IV, Table 11). There is little doubt, therefore, that the polysaccharide constituents of the O antigens determine type specificity and give rise to protective antibodies, and there is much evidence, not only from animal experimentation but also from extensive epidemiological analysis, that antityphoid vaccination has been rendered more effective by directing the immunization

technique toward the production of higher anti O titers (Boyd, 1943a; Callender and Luippold, 1943).

In addition to the classical O antigen, the strains of typhoid bacillus isolated from human pathological material possess another antigenic component, the Vi substance, which produces antibodies capable of protecting experimental animals against infection with strains containing this antigen. In fact, the possibility of obtaining virulent variants of *Eberthella typhosa* containing either Vi without O, or O without Vi, or both antigens together, has permitted the analysis of the protective value of the two antibodies. Either one of these antibodies is capable of protecting

TABLE 32

SUMMARY OF 4 INDIVIDUAL EXPERIMENTS PERFORMED IN AN IDENTICAL MANNER, IN WHICH THE IMMUNOGENIC CAPACITY OF BALLERUP V AND W, AND TYPHOID V AND W, VACCINES, TO PROTECT MICE AGAINST *S. ballerup* V AND *E. typhosa* V WAS COMPARED

TEST ORGANISM	DOSAGE OF ORGANISMS	Results are expressed as a fraction, the denominator of which indicates the number of each group of vaccinated mice subjected to the stated dosages of the test organisms, while the numerator indicates the number of these that died			
		PROTECTION OF MICE AFFORDED BY VACCINATION WITH:			
		Ballerup V vaccine	Ballerup W vaccine	Typhoid V vaccine	Typhoid W vaccine
<i>S. ballerup</i> (V)	10(6)	0/16	7/16	0/16	11/16
	10(7)	0/16	11/16	0/16	13/16
	10(8)	7/16	13/16	10/16	16/16
Totals		7/48	31/48	10/48	40/48
<i>E. typhosa</i> (V)	10(6)	0/16	15/16	0/16	13/16
	10(7)	1/16	16/16	1/16	15/16
	10(8)	9/16	16/16	3/16	16/16
Totals		10/48	47/48	4/48	44/48
Grand total		17/96	78/96	14/96	84/96

Data from Longfellow and Luippold (1943, Table 2, p. 208).

against infection with a strain containing both antigens, but anti Vi cannot protect against infection with a strain containing O alone, and anti O cannot protect against a Vi strain deprived of O antigen. There has been much argument concerning the comparative protective effectiveness of the two antibodies; it is unlikely, however, that an answer to this question can be made until adequate methods become available for the quantitative estimation of the amounts of antibodies present in the sera used in the comparative protection tests (Bhatnagar, 1943; Boivin, Izard, and Sarciron, 1939; Felix, 1938; Felix and Bhatnagar, 1935; Grasset and Lewin, 1937; Henderson, 1939).

Although the antigens O and Vi are always present in the strains of the typhoid bacillus virulent for man, they also occur in other Gram-negative bacilli, irrespective of their virulence, or of their complete lack of pathogenicity. Thus, immunization with strains of *Salmonella ballerup* and of *Escherichia coli* containing Vi antigen gives rise to antibodies capable of protecting against infection with the Vi strains of the typhoid bacillus (Table 32). Similarly, "simulated" salmonella vaccines prepared with strains of paracolony bacilli containing certain antigens of the Kauffmann-White classification afford definite protection against infection with the virulent salmonella types which contain these same antigens (Longfellow and Luippold, 1942, 1943; Luippold, 1942, 1944). These examples, and the similar case of the protection induced against pneumococcus infections by immunization with entirely unrelated bacterial or fungal species containing antigens exhibiting the same immunological specificity (Chapter IV, Table 12), illustrate once more that the possession of a particular chemical configuration, and not the virulence of the organism in which it occurs, determines the effectiveness of an antigen as a protective immunizing agent.

Immunization with Nonspecific Bacterial Antigens.—The obvious importance of the type specific antigens, and the ease with which they can be studied, has led to the comparative neglect of the other components of the bacterial cells which can also give rise to protective reactions. There is, however, much evidence for

TABLE 33

RESULTS OF TITRATIONS OF PROTECTIVE SUBSTANCES AND AGGLUTININ-CONTENT OF TYPHOID-, PARA A-, AND PARA B-IMMUNE RABBITS' SERA, IN REFERENCE TO *E. typhosa*
(summary of 3 individual tests)

IMMUNE SERUM	INFECTIVE DOSE OF <i>E. typhosa</i>	RESULT AT THE END OF 72 HOURS	AGGLUTININATIVE TITER (live organisms)
Typhoid	10(6) 10(7) 10(8) 10(9)	0/12 0/12 5/12 11/12	1 · 10,240
Para A	10(6) 10(7) 10(8)	0/11 0/12 11/12	1 · 320
Para B	10(6) 10(7) 10(8)	1/12 0/12 9/12	1 · 320
Control (1) normal rabbit serum (1 test)	10(2) 10(3) 10(4)	1/4 4/4 4/4	1 · 20
Control (2) virulence of test organism	10 10(2) 10(3)	2/12 9/12 12/12	MLD is 10(3) organisms

Data from Longfellow and Luippold (1942, Table 4, p 254).

a type of acquired immunity which transcends the limits of type differentiation, and which is effective for a whole bacterial group. Unfortunately, these nonspecific, protective antigens have rarely been identified by serological reactions *in vitro* and are apparent only in protection experiments in animals. The lack of *in vitro* tests has naturally retarded the development of adequate techniques for the preparation and chemical identification of these antigens; to some extent, it has also hindered the improvement of immunization procedures.

There is no doubt that immunization with one type of salmonella affords some protection against infection with organisms

TABLE 34

THE DEGREE OF IMMUNITY CONFERRED ON MICE BY TWO INJECTIONS OF GRADED DOSES OF PARA B VACCINE, TO *E. typhosa*, *S. paratyphi*, AND *S. schottmulleri*
(summary of 3 individual tests)

Dose of para B vaccine (administered twice, at 1-week interval, and expressed as number of organisms)	Results are expressed as a fraction, the denominator of which indicates the number of each group of vaccinated mice subjected to the stated dosages of the test organism, while the numerator indicates the number of these that died		
	10 MLD <i>E. typhosa</i>	10 MLD <i>S. paratyphi</i>	100 MLD <i>S. schottmulleri</i>
million			
250		10/11	1/11
50	0/12	11/11	0/11
5	7/12	10/11	0/11
0.5	11/12	11/11	6/11

Data from Longfellow and Luippold (1942, Table 3, p. 253).

of several other serological types (Longfellow and Luippold, 1942, 1943). In many cases this cross protection is due to the fact that the strains concerned possess in common certain of the O antigenic components which determine some immunological relationship. Since, however, protection against infection with S forms can also be obtained by using as immunizing agents nontype specific R variants which are deprived of any O antigenic constituent, it is clear that a certain amount of protective immunization depends upon cellular constituents other than the specific polysaccharide (De Kruif, 1921, 1922; Li, 1929; Maltaner, 1934). Protection against infection with anthrax bacilli has also been described following immunization with the R avirulent form of this organism (Schaeffer, 1936; Stamatin, 1937; Sterne, 1937).

It has been pointed out that the protection induced by vaccines prepared from R cultures is of a low order, depending perhaps upon some antitoxic immunity, and, indeed, a low degree of non-specific antitoxic immunity can be established against several strains of Gram-negative bacilli by immunization with any one of

the members of this group (Zabl and Hutner, 1944; Zabl, Hutner, and Cooper, 1943b). A report of the comparative effectiveness of S and R variants of the typhoid bacillus for the immunization of rabbits against experimental infection with the virulent form of this microbial agent hardly seems compatible with the view that the immunity induced by R cultures is only of an antitoxic nature. Both variant forms of the typhoid culture used in this particular study were found to be equally toxic for rabbits, but only the S form was endowed with invasive properties and could establish a carrier state in this animal species. Vaccination of rabbits by the intravenous route protected them against fourteen invasive doses of the S form and vaccines prepared from R cultures protected as well if not better than those prepared from the S variants, even though they did not induce the production of any specific agglutinins for the S forms (Maltaner, 1934).

Interesting information is also available in the case of natural and experimental immunity to brucella infections. Human beings who have developed immunity as a result of clinical or sub-clinical infection with one of the three species of brucella can freely work with the others without danger of becoming infected. It is also generally accepted, and has become practice in veterinary medicine, that active immunity against brucellosis can be established in adult cattle by injecting them while calves with live *Brucella abortus* of very low virulence (Huddleson, 1942). The importance of some non-type specific antigen in the production of immunity to brucella is also indicated by the fact that guinea pigs can be protected by immunization with a soluble fraction extracted from the live bacilli ground up in the cold, even though this antigen does not elicit the production of specific agglutinins (Huddleson, 1943). Conversely, injection of a fraction containing the type specific complete gluco-lipid antigen elicits the production of specific agglutinating antibodies in guinea pigs, but does not increase the resistance of these animals to infection (Stahl and Hamann, 1941).

The mechanism of antiplague immunity is still very obscure. The immunizing value of a strain of *Pasteurella pestis* appears to

be unrelated to its virulence, or to the dissociative phase, smooth or rough, in which it is used. It seems to be associated with the presence of an ill defined structure, the "envelope," which can be present in rough or smooth, virulent or avirulent variants. In any case, the epidemiological evidence indicates that a high degree of immunity in man can be established by vaccination with live cultures of avirulent strains, and that these avirulent variants retain their immunizing value for experimental animals even when killed by heat or antiseptics (Girard, 1943; Girard and Robic, 1943; Jawetz and Meyer, 1943; Otten, 1941; Schütze, 1939; Vincke and Janssens, 1942).

Although pneumococci offer striking illustrations of the importance of type specific immunity, there is evidence of a type of antipneumococcal immunity which does not depend on antibodies directed against the type specific capsular polysaccharides. Thus, the results of immunization of miners in the Transvaal seem to show a nonspecific element in immunity to pneumonia (Wright, 1942). From the experimental point of view, it is possible to establish in rabbits an appreciable degree of nonspecific resistance against infection with fully virulent encapsulated strains by the intradermal injection of suspensions of heat killed pneumococci of any type, either in the encapsulated or non-encapsulated phase (Table 35); this immunity is not related to the heterophile antibody. Nonspecific resistance can also be established in mice by both active and passive immunization (Day, 1942, 1944; Dubos, 1938b; Enders, Wu, and Shaffer, 1936; Goodner and Stillman, 1933; Kolchin and Gross, 1924; Street, 1942; Tillett, 1928; Yoshioka, 1923). Partial autolysis causes nonencapsulated pneumococci to release in solution a fraction, consisting chiefly of a nucleohistone, which is capable of eliciting nonspecific immunity in rabbits and mice. There is, however, no evidence that the nucleohistone itself is the immunizing substance, and, as in other cases of nonspecific immunity, no serological reaction is available to facilitate analysis of the problem (Dubos, 1938b; Thompson and Dubos, 1938). The fact that the opsonic action and the protective effect against infection of mice with

TABLE 35

ACTIVE IMMUNITY AGAINST INFECTION WITH PNEUMOCOCCUS TYPE I IN RABBITS
IMMUNIZED WITH R STRAINS
(Three rabbits immunized with S_{III} are also included)

NUMBER OF RABBITS	IMMUNIZED WITH	INFECTED WITH PNEUMOCOCCUS TYPE III	ROUTE OF INFECTION	NUMBER DIED	NUMBER SURVIVED
1	R _I	cc. 0.01	Intravenous	0	1
3	S _{III}	0.1 0.01	"	1* 0	0 2
8	R _I	0.1 0.01	"	1† 0	5 2
4	R _I	0.1 0.01	Intraperitoneal	1‡ 0	0 3
6	R _I	0.1 0.01	Intradermal	0 0	2 4
Total 22				3	19
9	Normal controls	0.000001 0.000001 0.000001	Intravenous Intraperitoneal Intradermal	6 1 2	0 0 0
Total				9	0

* Animal died 8 days after infection

† Animal died 5 days after infection

‡ Animal died 7 days after infection Controls receiving test dose of culture died within 36 hours

Data from Tillett (1928, Table I, p. 793).

type III pneumococcus' can be counteracted or adsorbed with preparations of the nonspecific somatic C polysaccharide extracted from the R variant of type I pneumococcus, indicates that this polysaccharide, or some other nonspecific impurity contaminating preparations of it, plays some part in nonspecific immunity (Tables 36 and 37) (Enders, Wu, and Shaffer, 1936).

The Importance of Surface Antigens for Protection.—In addition to the few cellular constituents which elicit the development of resistance—specific or nonspecific—against infection, there exist in bacterial cells a large number of different substances

TABLE 36

EFFECT OF SOMATIC POLYSACCHARIDE AND OF CAPSULAR POLYSACCHARIDE ON THE PHAGOCYTOSIS OF PNEUMOCOCCUS TYPE III BY THE NORMAL SERUM AND POLYMORPHONUCLEAR LEUCOCYTES OF MAN

STRAIN	AGE OF CULTURE	MATERIAL TESTED	AVERAGE NO OF COCCI PER 10 CELLS	CELLS CONTAINING COCCI	NO. CELLS COUNTED
	hrs.			per cent	
SV	18	—	61.0	94.0	50
SV	18	C 1:100	0.2	2.0	50
SV	18	SSS III 1:100	0.0	0.0	50
CH	18	—	221.6	100.0	50
CH	18	C 1:100	141.2	94.0	50
CH	18	SSS III 1:100	32.2	58.0	50

Normal
human
cells and
serum

Data from Enders, Wu, and Shaffer (1936, Table I, p. 427)

TABLE 37

THE OPSONIC EFFECT OF ANTI R PNEUMOCOCCUS SERUM AND THE INHIBITORY EFFECT OF THE SOMATIC C POLYSACCHARIDE ON THE PHAGOCYTOSIS OF PNEUMOCOCCUS TYPE III BY RABBIT LEUCOCYTES

STRAIN	AGE OF CULTURE	CONSTITUENTS OF THE SYSTEM	AVERAGE NO. OF COCCI PER 10 CELLS	CELLS CONTAINING COCCI	NO. CELLS COUNTED
	hrs.			per cent	
CH	6	Normal serum	5.1	9.0	200
CH	6	Normal serum + anti-R serum	10.2	17.0	200
CH	6	Normal serum + anti-R serum + C 1:1,000	1.7	3.0	200
CH	16	Normal serum	87.0	86.0	50
CH	16	Normal serum + anti-R serum	128.0	96.0	50
CH	16	Normal serum + anti-R serum + C 1:1,000	69.3	79.0	100
SV	16	Normal serum	7.6	18.5	400
SV	16	Normal serum + anti-R serum	15.2	34.0	400
SV	16	Normal serum + anti-R serum + C 1:1,000	5.1	15.0	400

Data from Enders, Wu, and Shaffer (1936, Table II, p. 429).

which give rise to antibodies detectable by agglutination or precipitation reactions, but which do not confer protective immunity. The flagellar antigens, for instance, appear to belong to this latter group, although there are a few investigators who believe that antilflagellar antibodies play some part in resistance to infection (Arkwright, 1927; Bhatnagar, 1935; Felix, 1924; Greenwood, Topley, and Wilson, 1931; Springut, 1927). Immunological analysis has also revealed the existence in the Gram-negative bacilli of a number of substances such as the R polysaccharide, the ρ antigens, the T and Q proteins, the nucleoproteins, etc., which, even though ill defined chemically, can be characterized by the fact that they elicit the production of antibodies (Chapter IV:2). None of these antibodies, however, affords any protection against infection (White, 1931, 1932, 1933). Similarly, the nucleoproteins of pneumococci and streptococci appear to be of no importance in protective immunity, even though they readily elicit antibody production (Avery and Morgan, 1925; Avery and Neill, 1925; Lancefield, 1941).

Since the majority of antibodies induced by the injection of bacteria, living or dead, or of bacterial products, are completely devoid of protective power against infection, it is of the greatest theoretical and practical interest to determine what attributes are required to determine a protective reaction. From this point of view, great importance has been attached to the location of the different antigens in the architecture of the bacterial cell, and it is generally held that those components of the cell which are situated at or near its surface are the most effective in eliciting protective immunity. This view has a definite factual basis, since some of the most effective antigens, such as the pneumococcus capsular polysaccharides, the type specific M protein of group A streptococci, the O and Vi antigens of Gram-negative bacilli, are undoubtedly situated at the periphery of the cell. It also possesses a certain logical character, since antibodies, being large molecular globulin proteins, cannot, or can only with difficulty, penetrate the plasma membrane of living bacteria and must be largely restricted in their action to the superficial cellular structures.

Granted these facts, it must be pointed out that the theory of the relation of surface antigens to immunity is only a first approximation, and does not define the actual phenomena with enough precision or completeness. It does not define, for instance, the nature of the "surface" concerned, whether it refers to the rigid cell wall which is an essential structure of all the bacterial species so far studied, whether the inner surface of this cell wall is as important as its outer surface, or whether the protective antibodies react with the plasma membrane which, in bacteria as in other cells, determines the permeability and osmotic properties, and which is probably the site of important metabolic reactions. The cell walls and plasma membranes are probably complex in structure, consisting of a mosaic of proteins, polysaccharides, lipids, etc., and it is unlikely that these different components are of equal importance in the immunity reaction, not only because they differ in antigenic activity, but also because their union with the antibodies may not in all cases result in an interference with the pathogenic process.

Ignorance of many of the essential factors of this problem precludes any complete analysis of the phenomena of immunity. It must be mentioned in this respect that several of the known superficial components of bacterial pathogens appear to be of little or no significance in eliciting protective reactions. In some cases, as with the capsular hyaluronic acid of streptococci, this is due to the fact that the substance is not antigenic because it is chemically identical with one of the normal constituents of animal tissues (Kendall, Heidelberger, and Dawson, 1937; Meyer and Chaffee, 1940). Other surface constituents of the bacterial cell, like the flagellar antigens, the T substance of group A streptococci (Lancefield, 1941), the complete polysaccharide antigen of brucella (Stahl and Hamann, 1941), the capsular polysaccharide of the mucoid variants of the salmonella (Morgan and Beckwith, 1939), readily elicit the production of agglutinating antibodies, but do not render the animals resistant to infection. It is also doubtful that the antibody directed against the capsular polypeptide of the anthrax bacillus is of much importance in immune pro-

tection (Ivánovics, 1938b). The fact that a bacterial antigen is located at the periphery of the cell is not sufficient, therefore, to render it of significance in eliciting immune resistance. Nor does it appear that only surface antigens can give rise to protective reactions.

Pathogenic bacteria release, either by secretion or as a result of autolysis, a variety of substances such as coagulases, fibrinolysins, spreading factors, leucocidins, etc., not to mention the classical toxins, which greatly affect the course of the pathogenic process (Chapter VI:3, 4). These substances condition the invasiveness of the parasite, and the antibodies directed against them exert indirectly an antibacterial effect.

In summary, it is not to be doubted that the location and orientation of the different antigens in the bacterial cell are of great importance in determining the protective value of the antibodies to which they give rise. There are many factors, however, which obscure the operation of the law according to which the most superficial cellular structures are also the most important in this respect. Present knowledge of cellular organization is far too primitive to allow prediction of the nature of the bacterial constituents and products which condition the pathogenic career of the parasite, and which are vulnerable to the immunity processes.

The Chemical Nature of Protective Antigens.—Finally, it is clear that the chemical nature of a given antigen cannot be used as an index of its ability to elicit resistance. The paramount importance of the capsular substances of pneumococci has focussed the attention of immunologists upon the role of polysaccharides in immunity processes, and subsequent discoveries have justified this interest, since a number of other polysaccharides have been found to condition the serological specificity, the virulence and the protective antigenicity of several other bacterial species. It is important to remember, however, that polysaccharides do not occupy a unique position in these reactions. In hemolytic streptococci of group A, for instance, the protective antibodies are directed against certain proteins (the M substances) which also determine type specificity, and there is little doubt that the analysis of the

immunity processes in other infections will reveal that many proteins can elicit protective antibodies. It must be remembered also that several of the bacterial polysaccharides give rise to antibodies which are entirely devoid of any protective value; one need only mention, for instance, the somatic carbohydrates of streptococci or of the anthrax bacillus (Ivánovics, 1938b, 1940a; Lancefield, 1941). The streptococci illustrate in a particularly interesting manner this lack of relationship between antibody production and protective immunity. In these organisms, the virulence of the different bacterial groups for certain animal species is correlated with the possession by the pathogens of certain polysaccharides which determine the serological specificity of each group (Chapter IV:2). However, in spite of the relation between specificity in virulence and immunochemical specificity, there is no evidence that the antibodies which react with the group specific polysaccharides are of any importance in eliciting protection against infection (Lancefield, 1933).

It is certain, therefore, that bacterial polysaccharides, even though possessed of serological specificity, are not necessarily protective antigens. The capsular substances of pneumococci owe their importance, not to their chemical nature, but to their biological activity and to their location in the cellular architecture. The search for an effective immunizing agent, or the measurement of immunity by serological reactions, should not be based on the assumption that certain classes of chemical substances—proteins, polysaccharides, lipids, etc.—constitute important antigens. From the point of view of protective immunity, the importance of an antigen does not depend on its chemical nature, but rather on its ability to elicit the production of antibodies capable of interfering with the pathogenic career of the parasite.

3. MECHANISMS OF PROTECTIVE IMMUNITY

Reaction of Antibodies with Bacteria and Their Products.—Although agglutination and precipitation are the most obvious and most extensively studied of the phenomena which occur when

bacteria or their soluble products are mixed *in vitro* with the homologous antibodies, there is no indication that these reactions play any part in the mechanism of immune resistance to infection, or in fact, that they occur at all *in vivo*. Agglutination or precipitation are only the secondary manifestations, conditioned by a multiplicity of physicochemical factors, of a more fundamental primary reaction which consists in the union between the specific part of the antibody molecule, and the radical of the antigen which determines its specificity (Boyd, 1943b; Landsteiner, 1944; Marrack, 1938). There is little doubt that this primary reaction does take place *in vivo*, and accounts for many of the phenomena of immunity. According to the nature of the test and the conditions under which it is performed, the "sensitization" of bacteria by the homologous antibody can result in phagocytosis by active leucocytic cells, or in bacteriolytic or bactericidal action if serum complement is added to the system. Immune bacteriolysis and phagocytosis are, like agglutination, only secondary reactions. These facts have led to the formulation of the unitarian theory, according to which the terms agglutinins, precipitins, bacteriolytins, opsonins, etc., are applicable to the same antibody tested under different conditions. It must be clear, however, that one bacterial cell can give rise to several antibodies, each corresponding to one of the antigenic determinant groups of this particular cell. The unitarian theory claims only that agglutination, precipitation, bacteriolysis, phagocytosis, etc., can all be the manifestation of one given antibody reacting with one specific determinant group of the bacterial antigen, but under different conditions of observation. The theory does not rule out the possibility that different antibodies, reacting with different chemical groups of a given cell, can cause these various reactions (Boyd, 1943b, p. 22; Zinsser, Enders and Fothergill, 1939, p. 175).

It is not always easy to reconstitute the chain of events by which the primary reaction between antigen and antibody results in the control of the infectious process. There is no well authenticated case where the protective antibody directly causes the death, or interferes with the metabolic processes, of the susceptible para-

site. In all known cases, inhibition or elimination of the bacteria requires the participation of certain host factors, such as phagocytic cells or complement. Furthermore, it is probable that antigen-antibody reactions account for only a part of the immunity process and that, in addition to antibody production, tissue cells have other ways of adapting themselves to the toxic action of bacterial products, or of interfering with the multiplication of the parasite. The increased production of certain enzymes by adaptation, the development of tolerance by modification of permeability or of certain metabolic processes, are perhaps of common occurrence, but have never been adequately studied. The possibility of interfering with the growth processes of the infective agent has been recognized, but has not yet been convincingly proven; even the reports of anablastic immunity in the case of protozoan infections have not remained unchallenged (Augustine, 1943; Taliaferro, 1931). As often happens, the growth of knowledge has been determined by the availability of simple experimental techniques (the serological reactions in the present case), rather than by the possible importance of different lines of investigation. It is therefore only for lack of other information that our survey of immunity processes will deal chiefly with the reactions of classical immunology.

Bacteriostatic and Bactericidal Action of Immune Antibodies.—Freshly drawn blood, even when deprived of its cellular elements, is a poor medium for the growth of bacteria; in fact, fresh serum or plasma is capable of killing appreciable numbers of cells of different bacterial species. There is little doubt that this bactericidal power manifests itself *in vivo* as well as *in vitro*, and accounts for some of the normal resistance exhibited by certain animal hosts for many microorganisms. Thus, a certain degree of correlation seems to exist between the resistance of rats to infection and the bactericidal power of their white blood cells (Irwin and Hughes, 1931, 1933). Nothing is known of the origin and nature of this bactericidal power, whether it is due to some normal physiological constituents of the serum, or whether it is the result of antigenic stimulation caused by the many types of substances and micro-

organisms which find their way into the tissues during life. In any event, it is certainly the expression of a multiplicity of serum constituents, since adsorption of the serum with one strain of bacteria, while decreasing somewhat the bactericidal power for all other strains, abolishes it completely only for the homologous ones (Gordon and Carter, 1932; Gordon and Johnstone, 1940). This nonspecific bactericidal power varies greatly under many different conditions; it increases rapidly (within a few minutes or hours) after injection of unspecific substances, and decreases again within a few days (Topley and Wilson, 1937, p. 920). There occurs also a marked rise in the bactericidal power of blood for certain bacteria (especially streptococci) during the febrile periods of a variety of diseases (Tillett, 1937; Wulff, 1934).

As a result of clinical or subclinical infections, or following the injection of adequately prepared vaccines, the bactericidal power of the serum may increase specifically with reference to the bacterial species which elicits the immune response. The immune bactericidal effect requires the participation of complement, a complex of several components of normal serum, which, by an unexplained mechanism, causes the lysis of many types of cells (Gram-negative bacteria, erythrocytes) sensitized by specific antibody.

The occurrence of the bactericidal action *in vivo* and its possible role in determining the resistance to infection of immunized animals has been repeatedly shown in the case of many species of Gram-negative organisms, and especially with cholera vibrios. These organisms rapidly undergo dissolution when injected into the peritoneal cavity of a cholera immune guinea pig. The action is specific and can be transferred from an immune to a normal animal by injecting immune serum together with the bacteria; in a normal animal so treated, lysis is in every way similar to that observed in the immune. Lysis of the vibrios results in the liberation of their endotoxins, but there is evidence that the resistance to infection resulting from specific immunization is due to the bactericidal effect of the serum and not to its ability to neutralize the toxin (Pfeiffer, 1894; Pfeiffer and Issaëff, 1894; Pfeiffer and Wassermann, 1893).

Although the R variants of many Gram-negative organisms are extremely susceptible to lysis by complement, the S forms of the same organisms are more resistant. Since the latter organisms differ from the former by the possession of the type specific O antigens, it appears possible that the O polysaccharide can inhibit in some way the bacteriolytic reaction. Indeed, the type specific antigenic substances extracted from organisms of the salmonella group inhibit markedly the bactericidal power of human and animal immune sera (Table 26) (Cundiff and Morgan, 1941). The type specific antigen of *Shigella dysenteriae* also inhibits the bactericidal power of normal rabbit serum for this organism (Table 25) (Thibault, 1939). This inhibitory effect can be specifically neutralized by the addition to the system of the homologous anti-serum, a fact which explains the marked increase in bactericidal power for the S forms which follows immunization. Quantitatively, the bactericidal reaction induced by complement and immune antibody behaves as a first order reaction, since whatever the number of bacteria used in the test, a constant percentage is killed by one given serum-complement system over a given length of time (Morris, 1943). Although the ultimate mechanism of the reaction is unknown, the experimental facts are compatible with the view that complement can attack the susceptible bacterial substrate only after the charges on the surface antigens of the cell have been neutralized by the homologous antibodies.

The Phenomena of Phagocytosis.—Whereas practically all species of Gram-negative bacteria are susceptible to the bactericidal effect of immune serum, the Gram-positive species are only little if at all affected by it. The phenomenon of phagocytosis, on the contrary, has been shown to occur with all types of micro-organisms and is considered by many to constitute the most important means of disposal of infective agents. In fact, it is well to remember that phagocytosis is not a process peculiar to infection, but is a general biological phenomenon (Metchnikoff, 1901). Phagocytic cells are capable of ingesting all sorts of foreign particles whether inert matter or bacteria, dead or living. There is then nothing remarkable in the fact that phagocytes can engulf

many types of bacteria; the real problem is to elucidate why certain microorganisms, the virulent forms in particular, are resistant to this action, and why specific antisera permit the phagocytosis of otherwise resistant forms.

Some of the facts of the problem are illustrated in the following experiment. Virulent streptococci injected intraperitoneally into a normal guinea pig multiply rapidly without any evidence of phagocytosis. If, on the other hand, a suspension of avirulent proteus bacilli is injected into the same animal at a time when the virulent streptococci are undergoing active multiplication, the leucocytes at once ingest the proteus bacilli in enormous numbers. Furthermore, when the virulent streptococci are injected into a guinea pig previously treated with antistreptococcal serum, the peritoneal exudate of the animal rapidly gives evidence of phagocytosis mainly by polymorphonuclear cells (Bordet, 1897; Marchand, 1898).

A complete understanding of the phenomenon of phagocytosis requires more complete knowledge of the nature and physiological activity of the phagocytic cells, as well as of the environmental conditions under which they function. It will be necessary to limit our analysis to the nature and properties of those components of the virulent bacteria which prevent them from behaving as inert particles toward the phagocytic cells of the host. As in the case of serum bacteriolysis, the R variants of bacteria are in general much more susceptible to phagocytosis than the smooth or mucoid type specific forms. Thus, phagocytic cells fail to take up anthrax bacilli which have developed capsules in the animal body, although phagocytosis readily occurs when bacteria of the same species are injected in the noncapsulated state as is found during growth in ordinary broth culture (Grüber and Futaki, 1907; Preisz, 1911). It is known that in streptococci and pneumococci, capsules are more readily demonstrated in young cultures, and that as a result of the destruction of the capsular material or of its dissolution into the medium, these structures become less and less evident as the culture ages. The fact that young encapsulated cultures are more resistant to phagocytosis than the older cultures

affords, therefore, an interesting correlation between encapsulation and phagocytosis (Table 28, Figure 29) (Chapter VI:2) (Hare, 1929, 1931; Seastone, 1934; Shaffer, Enders, and Wu, 1936; Ward and Enders, 1933; Ward and Lyons, 1935).

The possession of a true capsule is not indispensable to render a cell resistant to phagocytosis. Bacterial forms possessing type specific antigens can also be resistant even though they are not encapsulated; such is the case for the matt variants of hemolytic streptococci of group A. Similarly, the presence of the Vi antigen increases the resistance of typhoid bacilli to phagocytosis (Almon, 1943; Bhatnagar, 1935). It seems likely, therefore, that a certain class of substances, the type specific antigens in particular, possess to an especially high degree the power of interfering with the action of the leucocytes. The fact that these substances are concentrated around the bacterial cell during the early period of growth probably accounts for the greater resistance of the young forms to phagocytosis (Chapter VI:2).

It is possible that the inhibitory action of the capsule is not due merely to the fact that it constitutes a physical barrier, a mechanical interference between phagocytes and bacteria. The capsule should, perhaps, be regarded rather as corresponding to a high local concentration of a substance, the capsular material, which is endowed with physiological properties resulting in anti-phagocytic action. There is, indeed, convincing evidence that some of the specific antigens exhibit great antiphagocytic activity even when acting in solution. This can be demonstrated by observing the phagocytosis of pneumococci in serum leucocyte mixtures. In such a system, nonencapsulated pneumococci are readily engulfed even in the presence of normal serum; the addition of small amounts of solution of purified capsular polysaccharides is sufficient, however, to inhibit the phagocytic reaction (Sia, 1926). That the type specific antigen of *Eberthella typhosa* also possesses antiphagocytic activity is indicated by the pronounced negative chemotactic effect which it exerts on the migration of guinea pig leucocytes (Morgan and Upham, 1941).

Immune resistance to infection is correlated in many cases with

the appearance in the serum of antibodies which can promote specifically the phagocytosis of the bacterial species with reference to which resistance has developed. Here again, the specific antigens play a role of considerable importance in eliciting the production of these antibodies. Indeed, it appears that the slight opsonic activity of normal human and animal sera for virulent bacteria can also be due to antibodies reacting with these same cellular antigens. Thus, in the case of the typhoid bacillus, the opsonic effect of normal serum is due to the presence of small amounts of O antibody, whereas the normally occurring H (flagellar) antibody seems to have no effect on phagocytosis (Bhatnagar, 1935). The opsonic action of normal adult human serum for encapsulated pneumococci appears also to be due to antibodies directed against the specific capsular polysaccharides (Enders and Wu, 1934; Ward and Enders, 1933). As a result of the addition of the specific carbohydrates of pneumococcus types II and III, the ingestion of young encapsulated pneumococci by polymorphonuclear cells in fresh normal adult serum is specifically inhibited. Although the deacetylated form of type I pneumococcus is poorly active in this respect, the native acetylated material practically eliminates the opsonic action of normal human sera (Enders and Wu, 1934; Ward and Enders, 1933).

Since immunization with attenuated R variants can induce in some cases a marked degree of resistance to infection with the virulent forms, it is unfortunate that so little information is available concerning the effect of this kind of nonspecific immunization on the opsonic activity of the serum. Preparations of the nonspecific C carbohydrate obtained from cultures of an R variant of pneumococcus type I, inhibit the opsonic properties of the normal serum of man and the rabbit, which promote the phagocytosis of virulent type III pneumococcus. Furthermore, the antiserum prepared against the same R variant of pneumococcus type I has a definite, though limited, opsonizing effect upon the smooth type III organisms, which is abolished in the presence of a suitable quantity of the C carbohydrate (Tables 36 and 37). These findings are the more interesting in view of the fact that anti R pneumococcus

rabbit serum can protect mice against infection with type III pneumococcus, and that absorption of the serum with the C carbohydrate removes the mouse protective properties (Enders, Wu, and Shaffer, 1936). Although the C preparation used in these experiments was not a pure substance so that the results obtained with it can be due to some nonspecific impurity contaminating it, the observations retain their essential interest in demonstrating the existence in R pneumococci of a nonspecific antigen which can elicit immunity against the virulent forms and promote their phagocytosis (Chapter VII:2).

Mention should be made of the claims that bacteriophage can specifically increase the phagocytosis of susceptible bacteria. If, like bacteriophage lysis, the phagocytosis-promoting property of the lytic agent can be specifically inhibited by purified extracts of the susceptible bacterial cell, this phenomenon could become an additional technique for the identification of the bacterial components concerned in the phagocytic reaction (D'Herelle, 1926; Gerards, 1929; Nelson, 1928).

Phagocytosis is, undoubtedly, a most important mechanism of defense against infection. In the case of certain organisms, like the pneumococci, it probably results in the rapid death of the phagocytized cells. This outcome is not, however, a universal result of the process since there are many microorganisms which, like gonococci and meningococci, are adapted to intracellular life. Influenza bacilli also remain viable and capable of proliferation following engulfment, and staphylococci can be ingested in the presence of normal serum without being killed by leucocytes (Fothergill, Chandler, and Dingle, 1937; Lyons, 1937). Indeed, it has been observed that phagocytized typhoid bacilli are protected against the action of the bacteriolytic antibodies (Rous and Jones, 1916). In some cases, the development of immunity specifically increases the ability of the phagocytic cells to kill the bacteria which they ingest; thus, although toxigenic strains of staphylococci phagocytized in the presence of normal serum remain viable, they rapidly die if they are opsonized by immune antibody (Lyons, 1937). Similarly, ingestion of tubercle bacilli by the mononuclear phago-

cytes of normal animals does not affect the viability of the ingested bacteria; immunization or active tuberculosis, on the other hand, confer upon the mononuclear cells increased bacteriostatic or bactericidal properties, which appear to be independent of the immune body fluids and which remain manifest after these cells have been transported into a normal animal (Lurie, 1942).

Many factors must be considered in describing the relationship between the bacteria and the phagocytic cell: the negative chemotactic effect of certain bacterial constituents; the inhibitory effect of bacterial structures and the stimulating effect of certain antibodies on the engulfment process; the ability of the engulfed bacteria to survive in the intracellular environment, a property which in its turn is affected by the presence of antibodies, and of serum complement; the modification of some of the characters of the phagocytic cell itself as a result of immunization; the production by bacteria of toxins which kill the phagocytes, and the neutralization of these leucocidins by antibodies. It is clear, therefore, that although the observation of engulfment and its expression in terms of a phagocytic index reveal interesting facts concerning some phases of the phenomenon, many other elements must be taken into consideration in defining the importance of phagocytosis in the immunity process.

Antitoxic Immunity.—Classical immunology distinguishes sharply between antitoxic and antibacterial immunity, the former referring to a state of resistance to the poisonous products of the parasite, the latter concerning the ability of the host to get rid of the parasite or at least to prevent its multiplication. There are other characteristics which differentiate these two types of immune reaction. As far as is known, antibacterial immunity requires that a number of host factors such as serum complement and a complex system of phagocytic cells complete the reaction initiated by the antibodies. Antitoxic immunity, on the contrary, appears to be much more direct in its action, involving only the reaction of toxin with antitoxin; it is often more effective and final in its results provided the invasive power of the toxigenic agent is low enough to be controlled by the normal antibacterial mechanisms.

In most cases, however, antitoxic immunity is not sufficient to prevent death from infection with an invasive microorganism. Thus, a potent antitoxic serum capable of protecting rabbits against acute toxic death following the injection of large doses of hemolytic streptococci does not necessarily prevent fatal infection associated with multiple foci of infection (Parish and Okell, 1927). An antiserum active against the O streptolysin of hemolytic streptococci has no appreciable therapeutic action in mice (Todd, 1942). Rabbits immunized against staphylococcus toxin survive infection with living staphylococci longer than the controls, but eventually succumb to pyaemic infection (Burnet and Kellaway, 1930; Kellaway, Burnet, and Williams, 1930). There is no doubt, therefore, that the differentiation between antitoxic and antibacterial immunity is justified from many points of view. On the other hand, it will be pointed out presently that the two processes often overlap in their manifestations.

In addition to the classical toxins, pathogenic bacteria produce a number of different substances which, because of their biochemical and pharmacological activity, greatly affect the course of the infectious process. Thus, the spreading factors increase tissue permeability; the coagulases accelerate the clotting of blood plasma, the fibrinolysins dissolve the fibrin clot once formed; the leucocidins, hemolysins, and other cytotoxins kill or lyse certain of the host cells. These substances are not usually regarded as true toxins because their effects are not as dramatic as those of the classical representatives of this class of bacterial products. Still, they condition the invasive power of the infectious agent by virtue of their physiological activity, and the antibodies directed against them partake at the same time of antitoxic and antibacterial nature (Chapter VI:3, 4). It will be recalled, finally, that many of the bacterial structures against which antibacterial immunity is directed consist of substances which, like the complete antigens of Gram-negative bacilli, also exhibit physiological activity. The presence of these substances at the surface of the bacterial cell is responsible for the fact that virulent bacteria do not behave as inert foreign particles toward phagocytes.

One may wonder, therefore, whether, in many cases, antibacterial immunity is not in the final analysis the indirect expression of antibodies capable of neutralizing the toxic action exerted by bacterial constituents on the normal defense mechanisms of the host. Whether these active substances are to be called toxins, aggressins or cellular antigens, depends not only on their nature but even more on the point of view of the investigator. The phenomena of infection and resistance can be described in terms of the overall pathological effect of the infectious agent on the host, or they can be analyzed on the basis of the physiological and biochemical response of the tissue cells to each one of the constituents and products of the parasite.

4. PREPARATION OF IMMUNIZING ANTIGENS

Selection of the Antigen.—Of the many different antibodies elicited by the injection of a vaccine consisting of one single bacterial type, only very few exhibit a protective effect against infection. The rational development of immunization procedures requires therefore the identification of those singular cellular components upon which depends the protection reaction. Unfortunately, as we have seen, there is as yet no adequate basis for a general formulation of the problem. Protective antigens are not characterized by their chemical nature; whereas the capsular polysaccharides of pneumococci give rise to antibodies endowed with high protective effectiveness, the antibodies elicited by the somatic polysaccharides of streptococci, of anthrax, and of tubercle bacilli are completely inactive in this respect. Neither is the location of the antigen in the bacterial cell a satisfactory criterion of selection since, although both the M and T antigens of hemolytic streptococci of group A are surface constituents and type specific, only the former play an important role in the protection reaction. Not even a comparison of the antigenic structure of the virulent and avirulent forms of the same culture can serve as a dependable guide for the identification of the protective antigen since, in many cases, avirulent rough variants are very effective

immunizing agents (Chapter VII:2). Only by observation and experimentation, therefore, is it possible at the present time to define for each individual infectious agent the antigens which are effective in protective immunization.

Antigen versus haptene.—Granted that the proper culture has been selected and that serological tests are available to show that it contains the desired antigen, it does not follow that the vaccine prepared from this material will provide a satisfactory immunizing agent. The change from the antigen to the haptene state will serve to illustrate the many reactions, of physical, chemical, or enzymic nature, which can alter the antigenic efficacy of the vaccine.

Not all substances foreign to a host elicit the production of antibodies, *i.e.*, can function as antigen. This ability probably requires certain colloidal properties, a minimal molecular weight, and a number of other physicochemical characters, some of which, as a matter of fact, vary from host to host. The specificity of the antigen, on the other hand, depends upon some well defined molecular structure, which orients the antibody production and determines the union between antigen and antibody. It is therefore possible to destroy by chemical or other treatment a large part of the molecule of the antigen without destroying the groups or radicals which determine immunochemical specificity. These antigen fragments which have lost the ability to elicit the production of antibodies, but retain the ability to unite with antibody already formed, are called haptenes.

Thus, it is possible by alkaline treatment of cultures of cholera vibrios, of Gram-negative bacilli, or of pneumococci, to obtain purified polysaccharides which exhibit no antigenicity when injected into experimental animals, although they still react in high dilutions with sera obtained by immunizing horses or rabbits with the intact homologous organisms. An interesting example of loss of antigenic function is provided by the Vi antigen of *Eberthella typhosa*. This substance is extremely unstable to heat and other treatments, and exhibits a progressive loss of its immunological properties. The ability to inhibit the agglutination of typhoid bacilli by O antibody is the first property to be lost; then there disappear

in the following order: the ability to elicit production of mouse protective antibodies, to elicit Vi agglutinins in rabbits, to agglutinate in Vi antiserum, to absorb Vi antibodies from an antiserum (Almon, 1943). Understanding of the difference between antigens and haptenes is therefore essential for the preparation of immunizing agents. The fact that a bacterial vaccine exhibits a certain specific serological reaction reveals only the presence in the vaccine of the chemical group which determines specificity; it does not establish that this bacterial preparation will be effective as an immunizing agent, since the specific radical can have been changed to the hapten form instead of being present in the fully antigenic state.

Factors Affecting the Stability of Bacterial Antigens.—Bacterial antigens are organic substances, and it is not surprising that they are readily altered by many forms of drastic treatment. Exposure to alkaline reaction, for instance, modifies the immunochemical specificity, the biological properties, and in particular the antigenicity of the specific polysaccharide of type I pneumococcus (Avery and Goebel, 1933; Enders and Wu, 1934; Pappenheimer and Enders, 1933). We have mentioned how readily the Vi antigen of *Eberthella typhosa* is altered by aging, heating or treatment with all sorts of chemical agents (Almon, 1943). It is in the hope of minimizing the alterations suffered by unstable antigens that attempts have been made to devise "mild" methods for the killing of bacterial vaccines.

One of the chief objections to the use of heat for the sterilization of suspensions of bacteria is that this procedure results in the denaturation of proteins. In order to obtain "undenatured bacterial antigens," several authors have advocated the use of temperatures as low as compatible with adequate sterilization of the bacterial suspension. It is true, indeed, that certain bacterial components or products — such as many of the classical toxin proteins — are extremely susceptible to the effect of heat. There are, on the other hand, other antigens, like the capsular polysaccharides of pneumococci and the O antigens of Gram-negative bacilli, which are not protein in nature, and which are only little

affected by high temperatures; even some protein antigens such as the M substances and the erythrogenic toxin of streptococci are fairly resistant to heat. From the point of view of antibody production, therefore, the safe temperature at which a vaccine can be heated should be defined in terms of the particular properties of the specific antigen which is to be preserved in the active form. Nor is the lowest temperature compatible with sterilization of the culture necessarily the most desirable since, as we shall see, many antigens are inactivated by autolytic processes which are often favored by the use of lower temperatures during the course of preparation of the vaccines.

Chemical agents, such as phenol or formaldehyde, are often used for the sterilization of bacterial suspensions. In this case again, many antiseptics fail to inactivate completely some bacterial enzymes and therefore allow undesirable hydrolytic or oxidation processes to go on. Moreover, each particular antiseptic possesses chemical activities which, although innocuous for one given antigen, can be injurious to another. Thus, formaldehyde, when used under the right conditions, does not alter the specificity nor decrease the antigenicity of the capsular polysaccharides, and is therefore an ideal agent for the preparation of pneumococcus vaccines (Duhos, 1938a; Goodner, Horsfall, and Duhos, 1937). On the other hand, formaldehyde apparently impairs the ability of the Vi antigen of *Eberthella typhosa* to elicit the production of the mouse protective antibodies which are demonstrable in sera prepared with living bacteria (Felix and Bhatnagar, 1935). Formaldehyde may also affect the specificity of certain proteins, and should be used with caution when the antigen to be preserved belongs to this class of substances.

There have been many reports of the destructive effect of oxidation on some bacterial antigens. The streptolysin A and the hemolysins of the pneumococcus, the tetanus bacillus and the Welch bacillus, for example, are extremely susceptible to oxidative action although antigenicity is not always affected as fast as the hemolytic activity (Neill, *et al.*, 1926; Todd, 1932, 1934).

It is to be expected that, if allowed to act for a long enough

time, autolytic enzymes can profoundly alter the antigenicity of bacterial suspensions. Thus, prolonged autolysis of typhoid bacilli results in the enzymic breakdown of the complete antigen with conversion of the antigenic polysaccharide into a nonantigenic haptene. The problem of autolysis has achieved practical importance in the case of the pneumococci, organisms notorious for the ease with which they undergo autolytic breakdown. Death of these bacteria as a result of aging, slow heating, repeated freezing and thawing, desiccation by acetone-ether, lysis with bile and other surface active agents, treatment with acids or other antiseptics, etc., often results in the enzymic conversion of the capsular antigen into a polysaccharide haptene unless adequate steps are taken to prevent autolytic action (Chapter IV:3). Heat killing at low temperatures, a technique advocated by some workers to prevent protein denaturation, provides in reality conditions favorable for enzyme action, and results in a progressive alteration of the staining properties of the cells and in a loss of antigenic specificity. A better preservation of the specific antigen is obtained when the cells are rapidly brought to a temperature incompatible with enzyme action by a process of "flash heating." Even this technique is not without danger, however, since it is known that several enzymes are only reversibly inactivated by heat, so that in some cases enzymic action can be resumed after the cells have been brought back to lower temperatures.

When used under the proper conditions, formaldehyde, as already stated, is an ideal agent for the preparation of the specific antigen of pneumococci; it maintains intact their morphology, their staining reactions, their serological specificity and antigenicity. Interestingly enough, dissociation of the union between formaldehyde and bacterial cells, as can be obtained by washing the cell suspension free of the antiseptic or by bringing it to a slightly acidic reaction, permits some enzymic component of the autolytic system to recover its activity. When this happens, there occurs at the same time a transformation of the cell from the Gram-positive to the Gram-negative state, and a loss of specific antigenicity. It is possible to prevent these alterations from taking place, by main-

taining the treated bacterial suspension at an alkaline reaction which prevents the dissociation of the formaldehyde, or by heating the preparation at a temperature sufficient to cause irreversible inactivation of the enzyme (Dubos, 1937d, 1938a).

It is clear that no single method can be of general application in the preparation of immunizing agents; the method of choice will vary for each organism and more specifically for each antigen which it is desired to preserve in an active form. Heating of typhoid bacilli at boiling temperature does not deprive them of their ability to elicit the production of the antibody directed against the specific O polysaccharide, but this technique destroys the antigenic activity of both the flagellar and the Vi antigens. To preserve the latter it has been found advisable to kill the bacilli with ethyl alcohol without heat treatment (Felix, 1941). Just as no single method is applicable to all bacterial agents, similarly no one method is adequate for the preservation in their active form of all the multiple antigens of a given bacterial culture. In order to secure a complete expression in terms of specific antibodies of the antigenic mosaic of a cell, it would be necessary, therefore, to use several antigenic preparations of the culture, each designed for the preservation in its active form of one particular antigen.

Purified Antigens.—Since only one or a few of the antigenic constituents of a given bacterial species can elicit the formation of protective antibodies, it was natural to attempt to separate these effective antigens in a purified form, *free of irrelevant material*. The first achievements in this direction have been the use of culture filtrates for the production of antitoxins, and the later preparation in a purified form of well defined substances—the toxin-proteins of diphtheria and tetanus, for instance—which account for the biological activity of these culture filtrates. Among the substances which are capable of eliciting the classical type of antibacterial immunity, only the type specific O antigens of the Gram-negative bacilli appear to have been obtained in solution in a fully antigenic form. When injected into experimental animals or man, these soluble complexes are as efficient as the whole bacterial cells in eliciting the production of specific antibodies. The

soluble purified M proteins of group A hemolytic streptococci can induce specific precipitins and protective antibodies in mice and rabbits, but they are less effective in this respect than when still a part of the streptococcus cell. The complete capsular antigens of pneumococci have not yet been obtained in solution, although they can be freed of many irrelevant bacterial proteins by proteolytic digestion. The specific pneumococcus capsular polysaccharides themselves have been obtained in a pure, soluble form; even when purified without any drastic chemical treatment, however, they are devoid of antigenicity for rabbits and horses, although they can, under the proper conditions, elicit the production of specific antibodies in mice and men (Chapter IV:3).

The fractionation, isolation, and identification of the antigenic components of the bacterial cell thus constitute one of the main fields of immunochemical investigation. Techniques have also been developed to restore to polysaccharide haptenes their full antigenic activity. It has been claimed, for instance, that adsorption of the capsular polysaccharides of pneumococci on collodion particles renders them antigenic in rabbits (Zozaya and Clark, 1933). Although this finding has not yet been confirmed, it is compatible with the view that certain colloidal properties are a prerequisite of full antigenic effectiveness. In any event, it is well established that the capsular polysaccharide of type III pneumococcus can be rendered antigenic in rabbits by linking it with horse globulin; the protein confers upon the complex the physicochemical properties essential for antigenic function, while the polysaccharide determines immunological specificity.

In the complete antigens of Gram-negative bacilli, the specific polysaccharides occur in union with a conjugated protein; hydrolysis of the complex with acetic acid depolymerizes the polysaccharide but releases the conjugated protein in a native form; on the other hand, extraction of the antigenic complex with aqueous phenolic solution yields a highly viscous polysaccharide and denatures the protein. This purified viscous high molecular polysaccharide, while retaining all its serological specificity, is no longer capable of eliciting the production of specific antibodies in

rabbits; it is, nevertheless, still capable of combining with the native conjugated protein to form a complex endowed with full antigenicity. Of special interest is the fact that formation of this complex can occur even when the polysaccharide from one species of salmonella or shigella is added to the conjugated protein extracted by acetic acid hydrolysis from another species of these bacterial groups. In this case again, it appears that the protein is responsible for the property of antigenicity, whereas the polysaccharide determines specificity (Table 11) (Morgan and Partridge, 1942) (Chapter IV:2).

Synthetic Antigens.—We have repeatedly emphasized that the immunizing property of a bacterial vaccine depends upon some specific immunochemical group and not upon the bacterial cell as a whole. Striking illustration of this law has been found in the fact that protective immunity against *Eberthella typhosa* can be established with vaccines prepared from cultures of *Escherichia coli* possessing the same antigen as the former bacterial species; similarly it is possible to protect experimental animals against pneumococcus infection by means of antibodies prepared with microorganisms—the Friedländer bacilli, yeast, fungi, etc.—which, phylogenetically, are entirely unrelated to the pneumococcus group but which exhibit similar serological specificity (Tables 12, 32, 33, 34). In other words, the structure of its determinant immunochemical group, rather than its origin, determines the effectiveness of a given antigen as an immunizing agent (Chapter VII:2).

It should be possible, therefore, to produce protective immunity against a given infectious agent by means of an entirely synthetic artificial antigen possessing the proper immunological specificity; this experimental feat has been accomplished in the following experiments. The capsular polysaccharides of type III and type VIII pneumococci release on hydrolysis an aldobionic acid (cellobiuronic acid) which determines in part the serological specificity of these bacterial products. Artificial antigens prepared by combining the diazotized p-aminobenzyl β -glycoside of this acid with the globulin fraction of normal horse serum

elicit in rabbits the production of antibodies capable of conferring passive protection to mice infected with types II, III and VIII pneumococci. Moreover, rabbits immunized with this synthetic antigen acquire active resistance to infection with virulent type III pneumococci (Goebel, 1938, 1939). Similarly, antigens containing the azobenzylglycoside of the synthetic gentiobiuronic and glucuronic acids evoke in rabbits antibodies which protect mice against multiple lethal doses of virulent type II pneumococci (Goebel, 1940).

Thus, antigens prepared from materials which, from the point of view of their origin, are entirely unrelated to pneumococci can protect against infection with these organisms. They owe their activity to the fact that, because of their molecular structure, they can imitate the immunological behavior of those components of the pneumococcal cell which are responsible for eliciting the type specific protective reaction.

Immunization and Sensitization.—The practical procedures of immunization have not as yet been greatly affected by the knowledge available concerning purified and synthetic antigens. Identification of the cell components effective in bringing about protective immunity is too fragmentary, and the methods for their preparation in a fully active state are still too unsatisfactory, to permit practical application. It is worth pointing out, however, that although the antigens can be used in the form of killed bacterial cells for practical purposes, their preparation in the form of purified substances may eventually improve the practices of immunization.

It will be recalled that, in addition to the antigens concerned in protective immunization, bacteria contain a great variety of other components which give rise to the production of antibodies, devoid of protective power. The presence of these useless antigens is not a matter of indifference. The ability of the host to produce antibodies is not unlimited, since these substances are modified serum globulins and cannot occupy more than a definite proportion of the total serum proteins. When, as in emergencies, the need arises for the protective immunization of exposed popula-

tions to a multiplicity of infective agents—filterable viruses, rickettsiae, bacteria, protozoa, etc.—the possibility of overtaxing the antibody producing mechanism of the individual becomes a real problem. Elimination from the vaccine, by purification, of the useless antigens may therefore spare the body an unnecessary effort.

The production of antibodies devoid of protective power is not only an unnecessary load; it can have unfavorable and even dangerous results. Thus, immunization with pneumococcal and streptococcal cells elicits the production, not only of the protective antibodies directed against the capsular polysaccharides or the M proteins, but also of a variety of other antibodies, in particular those reacting with the ill-defined fraction known as nucleoproteins. Immunization is associated with an increased reactivity of the tissues to the bacterial products, so that the injection of minute amounts of the nucleoproteins, which would be without detectable effect in the normal animal, can produce violent necrotic and other toxic reactions in the immune. It is possible, therefore, that the antibodies to the nucleoproteins, although of no protective value, can in some cases affect unfavorably the clinical and pathological manifestations of the disease. It is in the case of tuberculosis that the greatest emphasis has been placed on the phenomena of hypersensitiveness, since injection of extracts of the tubercle bacillus into infected or sensitized animals produces violent reactions which can culminate in acute death.

Similar phenomena of hypersensitiveness have been recognized in many other bacterial infections and there has been much discussion concerning their relation to the immunity process (Rich, 1941, 1944). It is worth reiterating that, of the multiple antibodies which result from the injection of a suspension of bacteria, or of extracts of them, only one or a very few afford protection against infection, while all cause different types and degrees of hypersensitiveness. Each one of these antibodies affects in several directions the pathology, symptomatology, and outcome of the infectious process, and the analysis of such a complicated system is likely to face insuperable difficulties. Separation as individual

chemical entities of the different active components of bacteria, and study of the properties and behavior of these purified substances in the animal body, constitute essential steps in the analysis of the relation of sensitization to resistance. The knowledge thus acquired, and the availability of pure antigens, would undoubtedly help in the development of rational methods of immunization.

5. APPRAISAL OF IMMUNIZING EFFICACY

Immunochemical Methods.—Comparisons of the immunizing value of different bacterial antigens are beset with great experimental difficulties which account for the lack of quantitative formulation of the problem. The failure of the usual type of bacteriological and chemical information to describe the immunizing efficacy of a vaccine can be illustrated by comparing the antigenicity of two preparations of encapsulated pneumococci, obtained from the same culture, corresponding therefore to the same number of cells, exhibiting the same turbidity and giving the same gross chemical analysis. If the pneumococci in one case have been killed under such conditions that they remain morphologically well preserved and retain their Gram-positive staining character, they can elicit in rabbits or horses the production of type specific protective antibodies directed against the capsular polysaccharide. If, on the contrary, the bacterial suspension has suffered even a slight degree of autolysis during the preparative procedures, resulting in the loss of Gram-positiveness, it fails completely to exhibit type specific antigenicity in the same animal species and only gives rise to the production of group specific, nonprotective antibodies. This loss of specific antigenicity is the more remarkable since the type specific polysaccharide itself is still present in the latter bacterial suspension as can be demonstrated by serological reaction and chemical identification. Similarly, a different degree of heating of two otherwise identical suspensions of typhoid bacilli completely changes their ability to give rise to the anti Vi mouse protective antibodies, without de-

stroying the ability of the Vi antigen to react in specific antisera. It is clear, therefore, that the only convincing test of immunizing efficacy available at the present time is the response of the immunized animal.

In many cases, the development of resistance to infection is associated with the production of antibodies, the action of which can be recognized *in vitro*. The development of methods for the quantitative determination of antibodies is indeed from the intellectual, the theoretical, and the practical point of view, one of the great achievements of immunochemistry (Boyd, 1943b; Heidelberger, 1939). Needless to say, quantitative methods for the measurement of immunity are possible only on the basis of a fairly accurate knowledge of the specific antigen concerned in the protection reaction and there are, unfortunately, only few cases in which this knowledge is available. It should be remembered also that some of the phenomena which result in protection are not detectable *in vitro* by present methods, and that antibody reaction is probably only one of the many mechanisms of immunity.

Protection Tests in Animals.—In any event, *in vitro* methods of measurement of antibodies must always be checked against, and referred to, the actual performance of the immunity system *in vivo*. The variability of individual animals does complicate the evaluation of the results of immunization procedures, but it can always be corrected by the use of numbers large enough to permit statistical treatment of the data. There are, however, more essential difficulties which prevent a simple statement and measurement of the immunizing efficacy of an antigen. The property of antigenicity is not an absolute character, but is dependent upon the animal species and upon the experimental conditions used to determine it. Thus, the capsular polysaccharides of pneumococci, although inactive in horses and rabbits, readily give rise in man and mice to specific antibodies. Again, animals of different species immunized with the same bacterial suspensions can respond with the production of antibodies which differ in their molecular dimen-

sions, in solubility properties, even to some extent in their immunological specificities. The route of injection as well as the length of immunization are also of importance in determining the properties of the antibodies produced. Encapsulated pneumococci injected intradermally into rabbits fail to stimulate the production of the specific antibodies which result from the intravenous injection of the same antigens. In this case, the leucocytes mobilized at the site of injection produce an enzyme which, although it leaves the capsular polysaccharide itself unaffected, inactivates the polysaccharide antigen which is essential for the immunization of rabbits (Chapter IV:3).

Different Mechanisms of Protective Immunity.—Discussion of the many factors which affect the reaction of animal tissues to the injection of a given bacterial antigen, and which thus determine the antigenicity of this substance for a given animal under a well defined set of conditions, is outside the scope of this survey. It is necessary to emphasize again, however, that the ultimate expression of immunization is resistance to the infective agent. In this respect, the complex nature of virulence adds a new set of variables which complicate still further the measurement of the protective effect of the immunizing agent against infection. As shown in Chapter VI, the virulence of a culture is the summation of a number of independent attributes which determine the ability of the parasite to establish itself in the host, to resist its varied defense mechanisms, to multiply in it, to do damage to it. Immunity can be the result of processes directed against any one of these factors of virulence or against several of them. The particular immunity process which is most effective in any given situation depends upon the nature of the pathogen and of the host, and upon the set of conditions under which the infection is taking place. It is possible, for example, that the type of antibody most effective for prophylactic immunization is not the same as that most effective in serum therapy. In other words, it may be desirable to aim at different structures or functions of the infectious agent, depending upon the end to be achieved: to prevent

the parasite from gaining a foothold on the host, to interfere with its multiplication, to destroy it, or to neutralize its products after it has become established.

In addition to the antibodies which have been recognized by *in vitro* reaction, there may be others which have remained undetected because they can operate only in the internal environment of the host, or because they require the participation of some unknown tissue components. It is also possible that resistance to infection is in some cases the result of modifications of the tissue cells and that it involves changes in permeability, altered enzyme production, and other modifications of metabolism. The techniques and points of view familiar to the immunologist, rather than the nature of the problem and the relative importance of its different aspects, have determined the current lines of investigation of the immunity process. There were developed early in the microbiological era a number of methods, based on serological and immunochemical reactions, which are so rapid in their performance and which have yielded such useful information that they have directed the study of infectious diseases along somewhat narrow channels. It is likely that by returning to the main channels of physiological and biochemical philosophy, the study of immunity will achieve a more complete picture of the many reactions by which the host responds to the specific stimuli exerted by the different cellular components and products of the parasite.

VIII

BACTERIOSTATIC AND BACTERICIDAL AGENTS

The poison goes where neither the hand nor the eye can go. It penetrates the ultimate constituents of the organism . . . becoming a reagent of extreme subtlety for the dissection of vital elements. By means of the poison, Claude Bernard established his laboratory in the very midst of the animal economy.

ERNEST RENAN

1. REACTION BETWEEN THE CELL AND THE ANTIBACTERIAL AGENT

S*pecific Receptors and Nonspecific Physicochemical Reactions Involved in the Phenomena of Antisepsis.*—Paul Ehrlich believed that antiseptics and chemotherapeutic agents exert their antimicrobial action by combining with certain chemically reactive groups of the susceptible cells. These hypothetical cellular structures which he called receptors, and for which the different agents were supposed to exhibit specific affinities, were called arseno receptors, orthoaminophenol receptors, acetico receptors, etc., depending upon the chemical groupings with which they were assumed to react. Differential susceptibilities to the different toxic substances could thus be explained by postulating the existence in the susceptible cell of a sufficient number of "receptors" concerned in some essential metabolic function (Ehrlich, 1908, 1913).

For a long time, the inadequacy of the biochemical knowledge concerning cellular structure and metabolism prevented any definition of the nature of the receptors in accurate chemical terms; only in the case of the arsenicals was the suggestion made that their toxic effects might depend upon their affinity for reduced thiol groups (Ehrlich, 1909). The receptor theory was

kept alive especially by workers interested in the chemotherapy of infections caused by protozoa, a fact of some interest since much of Ehrlich's experimental work had been done with this group of organisms. A few bacteriologists also attempted to describe the phenomena of antiseptics in terms of the original theory, claiming, for instance, that there exist in bacteria certain structures—the α receptors—which are not essential to the life of the cell, and others—the β receptors—which are so necessary that their alteration by antiseptics results in bactericidal effect (Aoki, 1937).

In the meantime, new facts had been uncovered which appeared incompatible with Ehrlich's views concerning specific receptor reactions and which led to the temporary neglect of his theoretical concepts. It was pointed out that most of the antibacterial agents commonly used are toxic not only to bacteria, but to practically all living cells. Their behavior as general protoplasmic poisons emphasized, not their ability to react selectively with specific "receptors," but rather suggested a mechanism affecting protoplasm in general (McCulloch, 1936). The toxic action of chlorine, iodine, peroxides, etc., for all types of cells could be explained as due to oxidation of essential protoplasmic constituents, and the effect of heat, heavy metals, phenolic compounds, etc., was traced to protein denaturation. The fact that the death curve exhibited by bacterial cultures subjected to the action of a great variety of antiseptics appears to correspond to a first order reaction, and is similar in particular to the curve of denaturation of proteins in pure solutions, led to the assumption that the death of bacteria results from the effect of the lethal agent upon one particular protein of the organism (Rahn, 1929, 1932, 1934). The very high temperature coefficient of the killing effect, of an order comparable to that of protein denaturation, again emphasized the importance of this mechanism in the phenomena of antiseptics (Chick, 1908, 1910, 1912, 1930). Thus, antiseptic action appeared to be the result of nonspecific effects which could hardly be ascribed to the susceptibility of specific cellular receptors.

In recent years, the trend in the analysis of the mechanism of antibacterial action has reversed itself. Instead of concentrating their attention on the study of death rates in bacterial cultures, and on the formulation of the physicochemical laws of the phenomena of antiseptics, investigators have become more and more interested in the chemical reactions which take place between inhibitors and cellular constituents. This new point of view is, in a way, a revival of the receptor theory, but formulated in terms of the available knowledge concerning the chemical structure of the bacterial cell, and of the biochemical reactions and catalysts involved in the processes of nutrition, respiration and growth. In fact, it is this last aspect of the problem, namely, the effect of antibacterial agents on metabolic processes, which has gained prominence during the past few years. The startling achievements, both theoretical and practical, which have resulted from the study of the antibacterial effect of the sulfonamides, have led many to think that "the growth inhibiting effect of antiseptics in general might be due to a specific poisoning of some essential reaction exerted through an interference with the catalyst or essential metabolite" (Fildes, 1940a). It is obvious that, ultimately, any antiseptic action results in the alteration of essential metabolic events. It must be remembered, on the other hand, that in a number of cases, the initial lesion affects the morphological structure of the cell rather than its metabolic equipment. Thus, the detergents cause the disintegration of structural complexes by virtue of their great surface activity, the bacteriolytic enzyme lysozyme attacks some polysaccharide constituent of the susceptible cells and causes the latter to undergo lysis. A complete survey of the mechanisms of antiseptics and of chemotherapy should consider, therefore, not only the specific inhibitors of essential metabolic reactions, but also the enzymic and chemical reagents which can selectively alter or destroy vital morphological structures, as well as the nonspecific physicochemical reactions which affect indiscriminately the protoplasmic constituents of all living cells. The examples to be considered in the following pages have been selected, not by reason of their practical importance, but

because they illustrate different types of reactions which can inhibit bacterial growth or cause the death of bacterial cells.

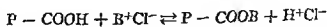
Formation of Un-ionized Complexes Between Dyes and the Basic and Acid Groups of the Cell.—The fixation of an ion by the cell is in a large measure reflected in its toxicity. Ions such as Na^+ and K^+ which are only weakly absorbed are not toxic in dilute solutions, whereas ions such as H^+ , Ag^+ or Hg^{++} are strongly absorbed and are toxic in dilute solutions (McCalla, 1941b) (Chapter III:1).

The theories which have been advanced to account for the remarkable antibacterial action of certain dyes are only a reflection of the attempts to define the mechanism of staining in terms of the forces which bind the stain to the cellular material. It may be said, in general, that the reaction of dyes with bacteria is an exchange reaction, the dye replacing similarly charged ions already present on the cell (McCalla, 1941a). In the case of acid dyes (e.g., acid fuchsin), the acidic ion is assumed to react with the basic groups of bacteria to form feebly-ionized compounds, giving rise to an ordinary type of double decomposition:



where P-NH_2 stands for some basic cellular component and A^- stands for the dye anion. Although this reaction would be favored at acid reaction, it must be pointed out that acid dyes can exert an antibacterial effect at pH values so low that the acidity of the medium is often incompatible with the growth of many bacterial species (Stearn and Stearn, 1924b).

The basic dyes (tryphenylmethanes and acridine compounds in particular) appear to owe their biological activity to their basic ions, and to form with the acidic groups of bacteria feebly-ionized compounds:



where $\text{P} - \text{COOH}$ represents an acid cellular constituent and B^+ the dye cation; this reaction is favored at alkaline reaction (Albert, 1942) (Chapter III:1).

According to this view, there is no essential difference between the reactions induced by dyes as stains and as bacteriostatic agents. Microorganisms show iso-electric behavior with reference to dye bacteriostasis as well as to staining. They are increasingly sensitive to basic dyes as the pH is increased, and to acid dyes as the pH is decreased; in fact, a culture completely inhibited in its growth by a basic dye may be caused to grow out, all other factors remaining constant, by decreasing the pH of the medium (Stearn and Stearn, 1924b, 1926, 1928b, 1930a, 1931b; Stearn, 1933). The antibacterial effectiveness of a dye, therefore, should depend in part upon the strength of its free acid or base. In the case of a basic dye, the bacteriostatic power should increase with the basic strength (within one series of closely related compounds), on the double assumption that the ionization of the antiseptic increases as the bases of which they are the salts become stronger, and that the antiseptics derived from strong bases form complexes more resistant to hydrolysis with the acidic groups of the cells. One could foresee, furthermore, that if the base became too strong, it might become less effective because it would be less able to form un-ionized complexes.

Some evidence for the direct relationship between basic strength and bacteriostatic activity can be found in the triphenylmethane series in which the antiseptic activity increases in the following order: aniline blue, magenta, crystal violet, malachite green and brilliant green, which also appears to be the order of increasing basicity. Unfortunately, the triphenylmethane series does not lend itself to direct determination of basic strength, but only permits indirect estimation of it. On the other hand, the basic strength of the different members of the acridine series can be determined directly by potentiometric titration, and it is possible to establish convincingly that passing from the weakest base (1-aminoacridine) to the strongest (5-aminoacridine) the antiseptic power increases progressively (Table 38) (Albert, 1942).

Factors other than basic strength do, of course, greatly affect the biological activity of dyes. Thus it has been observed that, as the basic strength increases in the acridine series, the com-

TABLE 38

CORRELATION OF BACTERIOSTATIS AND BASIC STRENGTH AMONG AMINO-ACRIDINES

SUBSTANCE	MEDIUM: 10% SERUM-BROTH (pH 7.2)					BACTERIOSTATIC INDEX (sum of inhibitory dilutions)	DISSOCIATION CONSTANT AT 25° C. (1×10^{-7})
	<i>Cl. welchii</i>	<i>Strep. haem. A</i>	<i>Staph. aureus</i>	<i>E. coli</i>	<i>Proteus vulgaris</i>		
5-Aminoacridine	7*	6	3	3	4	23	300
2:8-Diaminoacridine (proflavine)	7	6	3	3	2	21	120
2-Aminoacridine	7	4	3	4	3	21	12
4-Aminoacridine	5	2	1	1	0	9	0.1
3-Aminoacridine	5	2	1	0	0	8	0
Acridine	3	1	1	0	1	6	
1-Aminoacridine	2	1	1	0	0	4	

*Key to dilutions: 1 = 1 g. antiseptic prevents growth in 5000 ccm. Similarly, 2 = 1 in 10,000; 3 = 1 in 20,000, 4 = 1 in 40,000; up to 7 = 0 signifies no inhibition at 1 in 5000.

Data from Albert (1942, Table I, p. 635).

pounds became more hydrophilic and furthermore types of amino groups which basicity indicates—mined by their position in the molecule—may be of biological significance than basicity itself (Albert Rubbo, 1941). The acridine series also illustrated earlier, that the highest degree of basicity is not necessarily advantageous, since it does not form stable complexes of un-ionized complexes with the cell. The basicity of acriflavine (equal to that of sodium hydroxide) is nothing superior to proflavine, which is not (Albert, 1942).

The Effect of pH on the Antibacterial Substances.—Although the foregoing applies equally well to other

to those which owe part or most of their antiseptic action to surface activity. Thus, the neutral salts of higher aliphatic amines and of cationic detergents in general are antiseptics which correspond in some of their properties to the basic dyes (Randles and Birkeland, 1944). Instead of acid dyes we might have considered, as an example of anionic antiseptics, the neutral or faintly alkaline salts of other acids of high molecular weight, such as the soaps and other anionic detergents, ammonium and calcium mandelates, etc. The anionic compounds affect only the Gram-positive species when tested at neutral or slightly alkaline pH; Gram-negative organisms become susceptible to these substances at acid reactions. The cationic detergents are often more active and affect both Gram-positive and Gram-negative species over a wide range of pH although they are most active at alkaline reactions (Mallmann and Darby, 1941; Randles and Birkeland, 1944; Scales and Kemp, 1941; Valko and DuBois, 1944).

It has also been claimed that the phenols owe their antibacterial effect to a reaction between their hydroxyl groups and some cellular components. Since reactions which occur slowly in the bulk phase may occur rapidly when the reactants are oriented in a surface layer, and since phenolic compounds are surface active and therefore orient themselves at the cell surface, the reactivity of the phenolic hydroxy groups can become very great in biological reactions. Even in the test tube, phenols can precipitate amines provided one reactant is of sufficient molecular weight or carries a lipophilic constituent; it is not unlikely, therefore, that phenols combine with the amino groups of bacteria (Lahes, 1934). On this basis, phenols should be considered as anionic antiseptics, a theory which accounts for the fact that they are most effective at acid reactions.

In summary, it appears that acidic or basic high molecular antiseptics function by forming un-ionized compounds with vital groups in the bacterium. Such complexes, to be harmful to the cell, must not readily dissociate into their constituents. This requirement may account for the fact that surface activity and high

molecular weight, two properties which favor the formation of insoluble or feebly ionized complexes, are so commonly found in powerful antiseptics (Albert, 1942).

Competitive Inhibition of Enzymic Reactions.—The theories of antiseptics have undergone an evolution parallel to that of the theories of enzyme chemistry. With the symbol of lock and key relationship, Fischer introduced forcefully, as Ehrlich had done with the theory of specific receptors, the concept that the structural configuration of the reacting molecules conditions the reaction between enzymes and substrates. For a few decades, however, widespread interest in the phenomena of colloid chemistry led investigators to focus their attention upon the physicochemical aspects of the enzymic reactions which were explained in terms of surface chemistry. This type of approach revealed interesting facts concerning the effect of certain factors on the reaction rate. It failed, however, to account for the extraordinary specificity of the phenomena, and enzyme chemists found it necessary to return to the study of the specific reactions, expressed in terms of structural chemistry, which take place between enzymes and substrates. It has been found in particular that a given enzymic reaction can often be inhibited or blocked by substances which, because of the similarity of their molecular structures with that of the normal substrate, can react and combine with the enzyme, although they are not transformed chemically by it. Examples of competitive inhibition can be found in all fields of enzyme chemistry. Thus, xanthine oxidase can be poisoned by other purines which are not oxidized by this enzyme. Compounds such as thiamin or piperazine inhibit diamine oxidase. Amine oxidase similarly can be poisoned by amines which combine with it but are not attacked by it (*e.g.*, phenylisopropylamine or benzedrine) and there is reason to believe that the pharmacological effects of these amines are linked with their poisonous effects on the enzyme. Eserine combines reversibly with choline esterase, inhibiting its hydrolytic action on acetylcholine, a fact which is also of pharmacological importance.

Many examples can also be selected from the field of bacterial

chemistry. The dehydrogenation of lactate to pyruvate is inhibited by a series of compounds including α -hydroxybutyric acid and oxalic acid, but not by other related compounds which do not contain groupings in common with the substrates of the enzyme. Succinic dehydrogenase is similarly inhibited by compounds containing the group— CH_2COOH , notably by malonate, even though this compound is not "activated" by the enzyme, and in this case, enzyme inhibition can result in inhibition of growth. Certain bacteria can proliferate in a medium where the sole source of carbon is provided by succinic acid; if, however, malonate is also present in the medium, the growth of the bacteria is much retarded, as the malonate inhibits the oxidation of the succinate. Although malonate acts as an inhibitor of bacterial growth in this system, it is not a cell poison, for it does not act as an inhibitor in systems where a preliminary oxidation of succinate is not necessary to provide the bacteria with carbon for synthesis (Quastel, 1943; Quastel and Wooldridge, 1928).

Reactions Between Sulfonamides, Their Inhibitors and the Bacterial Cell.—The theory of competitive inhibition has found its most spectacular expression in the discovery that para-aminobenzoic acid can neutralize the bacteriostatic action of the sulfonamides, a fact which suggested that the latter substances owe their antibacterial effect to their ability to compete, because of their similarity in chemical structure, with para-aminobenzoic acid in some essential metabolic reaction (Woods, 1940). It is not our purpose to examine here the mechanism of action of the sulfonamides. In addition to p-aminobenzoic acid, a variety of unrelated substances such as methionine, urea, coenzyme, etc., can, under certain conditions, also inhibit the effect of the drug. On the other hand, homosulfanilamide (4-aminomethyl benzene sulfonamide) is insensitive to p-aminobenzoic acid and there are other unexplained observations which indicate that many details of the process remain to be clarified (Bliss and Deitz, 1944; Henry, 1943; Kohn, 1943a; Lawrence, 1944; Sevag and Green, 1944; Van Niel, 1943b). It is already possible, however, to formulate tentatively the laws which govern the interreactions be-

tween the drugs, their inhibitors, and the cellular structures for which they compete.

When suspensions of *E. coli* are treated with dilute solutions of p-aminobenzoic acid, sulfanilamide, and related compounds, it is found that all the chemotherapeutically active sulfonamides have an effect on the electrokinetic mobility of the organism resembling that produced by p-aminobenzoic acid, whereas the effect is quite different in the case of the inactive substances related to sulfanilamide. It appears that active drugs behave like p-aminobenzoic acid at the bacterial surface and that the association of the drug with the organism is a function of the aromatic amino group, a fact substantiated by other lines of evidence (Bradbury and Jordan, 1942; Green and Bielschowsky, 1942).

The chemotherapeutic activities of the different N¹-substituted sulfonamides are related to their acid dissociation constants. If one plots the pK^1 of the different compounds against their bacteriostatic activity, one obtains a smooth curve as the acid strength increases, passing through a maximum of biological activity at pK between 6.5 and 7. This correlation between acidic dissociation and biological activity is directly associated with the negative character of the sulfonyl groups, which is itself related to the electron attracting power of the N¹-substituent. The more negative the sulfonyl group, the greater the positive charge on the p-amino nitrogen, a fact of great importance since this amino group appears to be the one which combines with the organism. As the drug becomes more acid, the combination becomes less stable, and the antibacterial effect, therefore, less marked. Thus, for any particular pH there is one most active drug (at least *in vitro*, aside from clinical considerations of toxicity and absorption) characterized by the acidity of its $-\text{SO}_2\text{NH}-\text{R}$ group (Bell and Roblin, 1942) (Table 39).

Attempts have been made to describe the available data relating to the acidity of the different sulfonamides and their bacteriostatic activities in terms of law of mass action on the basis of the following considerations (Gaddum, 1943; Johnson, Eyring, and Kearns, 1943; Klotz, 1943). There is evidence that the

TABLE 39

DISSOCIATION CONSTANTS AND BACTERIOSTATIC ACTIVITY OF
SULFANILAMIDE TYPE COMPOUNDS

Compound	K_a	C_R Molar $\times 10^5$
N ¹ -Chloroacetylsulfanilamide	1.6×10^{-4}	10.0
N ¹ -Acetylsulfanilamide	4.2×10^{-6}	0.7
N ¹ -Methylsulfanilamide	1.7×10^{-11}	30.
Sulfanilamide	3.7×10^{-11}	20.0
N ¹ -Pbenylsulfanilamide	2.5×10^{-10}	3.0
Sulfapyridine	3.7×10^{-9}	0.6
Sulfadiazine	3.3×10^{-7}	0.08
Sulfathiazole	7.6×10^{-8}	0.08

C_R = minimum molar concentration necessary to cause bacteriostasis of *E. coli* at pH 7 in synthetic medium of standardized condition.

Data from Bell and Roblin (1942, Table I, p. 2906).

potency of a sulfonamide is a direct function of its protein combining capacity (Davis, 1943; Davis and Wood, 1942). If D stands for the sulfonamide ion combining with the protein P (a constituent of the bacterial cell in the present case), the concentration of PD is a measure of bacteriostasis. The relation of the pK of the sulfonamide of maximum activity to the pH of the solution can be expressed by the following formula:

$$pK_{HD} = pH - \log \frac{1-f}{f} \quad (1)$$

where

$$f = \frac{d \ln K_{PD}}{d \ln K_{HD}} \quad (2)$$

K_{PD} being the dissociation constant of the protein-sulfonamide complex. When f is determined for a given bacterial system, pK_{HD} can be predicted from the formula. Quantitative treatment of this type permits the prediction that among sulfonamides of

widely varying pK , the most effective compounds in producing bacteriostasis will be the ones of intermediate pK .

Although data for the direct evaluation of f for bacteria are unavailable, f can be evaluated indirectly for *E. coli* by the following method. In a solution at pH 7, maximum bacteriostasis of this organism occurs with a sulfonamide with a pK of about 6.7; substituting the appropriate values in (1), one obtains $f = 0.3$, a figure which compares well with the value (approximately 0.5) derived from the combination of serum albumin with sulfonamide (Davis, 1943; Davis and Wood, 1942). The inhibition of sulfonamide action by *p*-aminobenzoic acid is also amenable to a similar treatment. The theoretical prediction that the ratio of total amount of *p*-aminobenzoic acid necessary to cause inhibition, to the total amount of sulfonamide present, is at a maximum for the compound of greatest antibacterial activity, agrees well with observed facts (Rose and Fox, 1942).

Thus, the law of mass action not only permits the quantitative description of the reaction between the bacterial cell, the sulfonamide, and its inhibitor. It predicts also the existence and acid dissociation constant of a drug of maximum potency, it correlates biological activity with ionization constants, and it accounts quantitatively for the inhibitory effect of *p*-aminobenzoic acid (Klotz, 1943).

2. BACTERIOSTATIC VERSUS BACTERICIDAL EFFECT

Reversibility of Antibacterial Action.—The first and essential step in the action of many, if not all, antibacterial agents consists in a chemical combination between the agent and a cellular component of the culture under consideration. In certain cases, at least, this reaction between inhibitor and homologous cell substrate obeys the law of mass action and can be expected to be reversible under the proper conditions. Modifications of the acid base conditions in the environment, removal of the antibacterial agent, addition of substances exhibiting a great affinity for it, should facilitate the dissociation of the complex formed between

the inhibitor and the cellular substrate, and theoretically should restore the cell to a condition where growth is again possible.

Indeed, reversibility of the action of antiseptics assumed to exert a bactericidal action has been obtained in many cases. Cultures inhibited in their growth by the presence of a basic dye in a medium at slightly alkaline reaction can be caused to grow out by rendering the medium more acid, and thus decreasing the affinity of the dye for the acidic constituents of the cell (Stearn, 1933; Stearn and Stearn, 1928b, 1931b). Living bacteria stained and inhibited by electropositive dyes are decolorized when placed in certain organic media (broth, pus, serum, etc.) and recover at the same time their viability, provided decolorization occurs early enough (Doladilhe and Guy, 1940). Bacteria "killed" with mercury can be caused to multiply again following treatment with soluble reduced sulfur compounds (H_2S , glutathione, thiolactic acid, cysteine, etc.), which exhibit great affinity for the metal. The phenomenon can be demonstrated with particular ease in the case of bacterial spores which, probably on account of their slow metabolism, can regain their viability after prolonged exposure to mercury when treated with sulphydryl compounds (Clark, 1937; Engelhardt, 1923, Fildes, 1940b; Gegenbauer, 1922; McCalla, 1940). Another remarkable illustration of reversibility is found in the inhibition of growth of yeast and of *Lactobacillus casei* by a crystalline basic protein extracted from wheat. Although treatment of these microorganisms with the protein renders them unable to multiply, growth can be reinitiated by the addition to the system of different phosphatides. Since the basic protein is known to occur in wheat in combination with a phosphatide, one may assume that the reversal of antimicrobial action by the phosphatide is due to the formation of a lipoprotein which helps in dissociating the basic protein from the cell (Balls, Hale, and Harris, 1942; Woolley and Krampitz, 1942).

Factors Affecting the Stability of the Combination Between Inhibitors and Bacteria.—As already mentioned, the pK of the inhibitor and the pH of the medium condition the stability of the cell inhibitor complex. It is probable that the molecular size

of the antiseptic is also of some significance in determining the reversibility of its combination with the cell, since the property of high molecular weight favors the formation of insoluble or feebly ionized complexes (Albert, 1942).

On the other hand, many anionic and cationic antiseptics which contain in their molecules long lipophilic side chains, exhibit marked bactericidal activity on a great variety of bacteria. These compounds, commonly known as detergents, immediately inhibit the metabolic activity of the susceptible organisms, and their action appears completely irreversible, except if interrupted during the very early phase (Valko and Dubois, 1944). This irreversibility is still the more striking in view of the fact that the bactericidal effect of the detergent can be prevented by the addition of minute amounts of phospholipids to the system prior to the addition of the antiseptic, a situation to be contrasted with the ready reversal by a number of phosphatides of the antimicrobial action of the small molecular basic wheat protein mentioned above (Baker, Harrison, and Miller, 1941; Woolley and Krampitz, 1942). It has been suggested that the lack of reversibility of the antibacterial action of detergents is due to their great surface activity. These compounds congregate at the cell surface by virtue of their nonpolar groups which are made up of a carbon chain containing hydrogen and halogens, and which are repelled by the water. It is of interest to note in this respect that the phenolic antiseptics, typical bactericidal agents, are also surface active. In fact, it appears that, in the phenol series, the most powerful antiseptics are also the most surface active and inspection of the formulae of some 240 phenols suggests that bactericidal properties increase as the molecule assumes definite detergent characteristics (Table 40) (Suter, 1941).

The arsenicals which exhibit the greatest activity *in vitro* against trypanosomes are of the type phenylarsenoxide and xylylarsenoxide, substances which possess in addition to the hydrophylic arsenoxide group, a hydrophobic group which probably causes them to be taken up at some lipid-water interface of the cell (King and Strangeways, 1942).

TABLE 40

CORRELATION OF DISINFECTION AND SURFACE ACTIVITY AMONG PRENOLS

SUBSTANCE	PRENOL COEFFICIENT		SURFACE TENSION OF A 0.01% AQUEOUS SOLUTION (dynes per cm)
	<i>Staph. aureus</i>	<i>B. typhosus</i>	
Resorcinol	0.3	0.3	76
4-Propyl-resorcinol	37	50	73
4-Butyl-resorcinol	10	22	66
4-Amyl-resorcinol	30	33	60
4-Hexyl-resorcinol	98	50	51
4-Heptyl-resorcinol	280	30	43
4-Nonyl-resorcinol	960	0	Not recorded

Data from Albert (1947, Table II, p. 635)

It appears, therefore, that large molecular size and especially surface activity are properties which tend to endow antibacterial agents with bactericidal activity, by facilitating the formation of nondissociable complexes between the antiseptic and the susceptible cellular substrate.

Nature of the Difference Between Bacteriostatic and Bactericidal Effect.—The ultimate effect—bacteriostatic or bactericidal—of a given antiseptic is conditioned by a great variety of environmental factors among which can be mentioned the composition of the medium, its reaction, the temperature at which the test is carried out, etc. Of obvious importance is the nature of the microorganism involved. Group D streptococci are completely inhibited in their growth by minute amounts of gramicidin, but their viability is not affected by even maximal concentrations of the substance; bacterial suspensions maintained for several weeks in contact with the drug contain the same number of viable cells as untreated control suspensions, with the only difference that the growth of the treated cells is somewhat delayed. Pneumococci, on the contrary, are rapidly killed by gramicidin, and only by removing them very early from contact with the drug is it possible to differentiate the bacteriostatic from the bactericidal effect. The nature of the microorganism influences the outcome

of the antiseptic test, not only by determining the strength and stability of the combination between antiseptic and susceptible cellular substrate, but also because, during bacteriostasis, there occur within the cell certain secondary changes which result in irreversible alterations and therefore in death. The ease with which pneumococci undergo autolysis as soon as the medium becomes unfavorable for growth and multiplication accounts, in part at least, for the fact that many antiseptics which are only bacteriostatic for other more resistant organisms exert upon pneumococci a bactericidal effect. Analysis of the comparative death rate of pneumococci, streptococci, and staphylococci treated with penicillin reveals that, in this case again, pneumococci die more rapidly than the other organisms (Hobby, Meyer, and Chaffee, 1942). The occurrence of secondary irreversible alterations during bacteriostasis may also explain the observation that the bacteriostatic effect of the sulfonamides can be converted into a bactericidal effect by raising, from 37° C. to 40° C., the temperature at which the reaction is taking place (White, 1939). It is likely that, at the higher temperature, the rate of the catabolic reactions is increased while the compensatory anabolic reactions are inhibited by the drug. The more rapid exhaustion of some reserve substances, or the destruction of some essential structure, may be the direct cause of the bactericidal effect.

It appears, therefore, that the difference between bacteriostatic and bactericidal effect is often of a quantitative rather than of a qualitative nature. It depends upon factors which affect the rate at which irreversible alterations go on within the inhibited cell; it is conditioned by the firmness of the combination between the toxic agent and its "receptor" in the susceptible cell, and by the ease with which the combination can be dissociated. In fact, in order to demonstrate the bacteriostatic effect of a given antibacterial agent, techniques must be available to permit the dissociation of the complex formed by the antiseptic and the cellular substrate, before any irreversible alteration has taken place within the inhibited cell.

3. DIFFERENTIAL SUSCEPTIBILITIES OF DIFFERENT BACTERIAL GROUPS

Factors Affecting the Susceptibility of an Organism to a Given Antiseptic.—It is to be expected that bacterial cells, varying as they do in chemical composition and morphological structure, also vary greatly in their affinity for different chemical groups, and therefore in their susceptibility to different antibacterial agents. In this respect, the molecular configuration of the antiseptic is of obvious importance. In general, in any series of compounds wherein the change in structure involves only the length of the carbon chain, the lower members have little or no activity; the activity increases to reach a maximum, and then rapidly decreases as the number of carbon atoms increases in the carbon chain. Increase of activity in an ascending series appears correlated with a decrease in water solubility; an increase in lipid solubility, and, to some extent, an increase in surface activity. The loss of activity observed beyond the optimum carbon length is probably related to the limiting water solubility. Questions of solubility, partition coefficients, dissociation at a given pH, hydrogen bond formation, resonance, and other physicochemical properties are certainly of importance in conditioning the effectiveness of the antiseptic. Unfortunately, the information available on these aspects of the problem cannot as yet be interpreted in terms of cellular structure and is therefore beyond the scope of our discussion (Daniels, 1943).

In fact, there are only very few cases in which the chemical composition of the cell can be correlated with susceptibility to a given agent. The bacteriolytic enzyme lysozyme has been shown to hydrolyze certain acetyl aminopolysaccharides, and one can conclude, therefore, that bacteria susceptible to it contain substances of this type as essential parts of their structure (Chapter IV:1). Although the role of complement in the phenomenon of immune bacteriolysis is not yet understood, the fact that lysis requires, in addition to complement, the participation of specific

antibody, emphasizes the importance of specific cellular components in the killing effect.

The mere presence in the cell of a substrate exhibiting affinity for the antibacterial agent is not sufficient to render the cell itself susceptible to the latter. The susceptible cellular constituent must be so situated that it can be reached by the injurious agent. Cellular architecture, cellular permeability, and solubility of the agent should therefore be discussed in terms of each other. It may be pointed out at this time that phospholipids can protect living cells against many types of injurious agents; they have been reported to weaken the antiseptic action of mercuric chloride, phenol, and salvarsan on anthrax bacilli, and to inhibit the action of staphylococcus bacteriophage and of various bacterial lysins and toxins; they also reduce the cytolytic and hemolytic effect of saponin. It has been shown in particular that lecithin, cephalin, and sphingomyelin prevent the inhibition of bacterial metabolism, and the bacteriostatic and bactericidal effects caused by anionic and cationic detergents as well as by gramicidin (Baker, Harrison, and Miller, 1941). Extensive investigations of this phenomenon with gramicidin has established that, among phospholipids, the cephalins are by far the most active inhibitors. Their inhibitory effect can be observed not only *in vitro*, but also *in vivo*, and intraperitoneal injection of small amounts of cephalin completely prevents the protective effect of gramicidin against pneumococcal infection in mice. The amounts of cephalin required are exceedingly small, in fact, no larger than the amount of gramicidin employed. Since phospholipids are located at or near the cellular surface, they may be of significance in modifying the susceptibility of certain organisms to antiseptic action, a possibility which will be discussed again later.

There are many other factors which condition the resistance of a given culture to a given antibacterial agent. Thus, young cells are in general more susceptible than adult or old cells to injurious agents and procedures (Chapter V:2). Certain organisms overcome the inhibition of growth due to substances which interfere with some metabolic reaction, by utilizing an alternate metabolic

channel, or by increasing the efficiency of the inhibited step. Most microorganisms can give rise, under a variety of conditions, to a number of variant forms which differ profoundly from the parent strain in their susceptibility to one or more toxic agents (Chapter V:4). Several of these factors will be considered later in the analysis of the mechanism of drug fastness, and the present discussion will be limited to the striking correlation which exists between the staining properties of bacteria and their resistance to a variety of bacteriostatic and bactericidal agents.

Factors Affecting the Resistance of Acid Fast Bacilli to Toxic Agents.—The extraordinary resistance of acid fast organisms to most chemical antiseptics gives them a unique position among other living cells. It is not likely that this peculiar property resides in the protoplasm or is due to some unusual type of metabolism. As far as is known, acid fast bacilli carry on their metabolic processes through the agency of the same class of biologically active proteins, of enzymes in particular, which operate in other living systems. These active proteins exhibit a normal susceptibility to heat, as can be shown by the fact that both from the viewpoint of loss of viability and of metabolic activity, the thermal death point of acid fast bacilli is well within the usual range. Moreover, proteins extracted from these organisms can be rapidly denatured and rendered inactive by all usual toxic agents, acids and alkalis in particular. It is therefore extremely surprising that these same protoplasmic constituents can retain their characteristic properties and activities when the living cell is exposed to powerful denaturing agents, or to other antiseptics, aqueous solutions of 5% NaOH or H_2SO_4 , for instance.

These considerations suggest that the low susceptibility of acid fast organisms to chemical agents is not due to an unusual resistance of their metabolic constituents, but rather to a peculiar property of the cell which prevents injurious substances from reaching the zones of metabolic activity. The fact that no striking change in the rate of CO_2 production by tubercle bacilli can be observed between pH 4.4 and 7.4, whereas the metabolism of other bacteria is much more sensitive to reaction changes,

seems to confirm that the former organisms are not readily permeable to H^+ ions (Brooks, 1922; Loebel, Shorr, and Richardson, 1930; Richardson, Shorr, and Loebel, 1931).

It is tempting to assume that the lack of permeability of the acid fast cells to many toxic agents is due to the large amounts of a variety of lipids which they contain but, as already pointed out, there is no evidence that these lipids are organized around the cell in the form of a capsule (Chapter II:4). Although the surface of the acid fast cell possesses hydrophobic properties, the presence of this hydrophobic barrier is not sufficient to prevent the passage of water-soluble substances into the cell, since sugars, amino acids, and other water-soluble nutrients are readily utilized by tubercle bacilli. Moreover, these organisms are, despite their fatty structure, much more susceptible to water-soluble antiseptics than to fat soluble compounds. Indeed, fat solvents like benzene, toluene, xylene, chloroform, carbon tetrachloride, exert but little action against them, whereas certain water soluble substances like azo-dyes, neutral red, acridine orange, and a variety of other basic heterocyclic compounds are more active *in vitro* against tubercle bacilli than against non acid fast organisms (Hesse and Meissner, 1931; Lewis, 1916, 1917; Schöhl, 1924; Wells, 1932; Wells, De Witt, and Long, 1923). It appears, therefore, that to establish or disprove any causal relationship between the abundance of cell lipids and the resistance of acid fast organisms to chemical antiseptics, it will be necessary to gain a better understanding of the permeability of microorganisms to a variety of inorganic and organic substances.

Correlation Between Susceptibility to Toxic Agents and Behavior Toward the Gram Stain.—The Gram-staining technique divides the bacterial world into two groups which differ profoundly not only in staining characters, but also in other physico-chemical and biological properties, particularly in their resistance to many antiseptics (Chapter III:3). Since doubts have been expressed concerning the validity of this correlation (Waksman and Woodruff, 1942), it is necessary to emphasize again that so many unrelated factors can affect the susceptibility of a given

strain to an injurious agent that any general law of the phenomenon is necessarily obscured by a number of exceptions. It is not sufficient, for example, that a substrate capable of reacting with the inhibitor be present in the cell under consideration; it is also essential that this substrate be reached by the inhibitor, a condition controlled by properties of permeability. On the other hand, a given cell can perform its essential living processes through two independent metabolic channels, and can therefore keep on multiplying in the presence of an inhibitor effective against an important metabolic reaction. Finally, and perhaps most important, the reaction to the Gram stain reveals differences which are of a quantitative rather than of a qualitative nature. These differences may depend, as we have seen, upon the acid base properties of the cell material, or upon the permeability of the cell membrane to the reagents used in the staining procedure; in any event they correspond to a continuous series rather than to two absolute classes (Stearn and Stearn, 1931b). With these limitations in mind, it is justified to claim that, in general, most toxic agents exhibit a selective activity against either Gram-positive or Gram-negative bacteria, and that only a few are equally active against both groups of microorganisms. A brief survey of the behavior of certain classes of antiseptics towards the two groups of organisms will be presented in the following paragraphs to substantiate this claim.

Gram-positive organisms are inhibited by concentrations of gentian violet which permit the growth of Gram-negative bacilli (Churchman, 1912; Churchman and Siegel, 1928). This correlation holds true not only for other dyes of the triphenylmethane series, but in general for other strongly basic dyes, and in particular for those of the acriflavine group (Albert, 1942; Albert, Goldacre, and Rubbo, 1941; Browning, 1930; Browning, Kenaway, Gulbransen, and Thornton, 1917; Browning and Russ, 1917; Rubbo, Albert, and Maxwell, 1942). When bacteria are sensitized with erythrosin or safranin and exposed to both long and short wave lengths, the Gram-positive microorganisms show a much greater degree of sensitivity to the long wave lengths

than do the Gram-negative species (Dreyer and Campbell-Renton, 1936; Tung, 1938). Many salts are more toxic to the Gram-positive than to the Gram-negative organisms, and this relation holds true not only for particular salts, but also for their constituent anions and cations. On the whole, it appears that in contrast with strong electrolytes, weak electrolytes exhibit selective toxicity for Gram-positive bacteria (Smith, 1922).

The antiseptic anionic and cationic detergents are much more active against the Gram-positive than against the Gram-negative species; in fact, organisms of the latter group are completely resistant to the anionic compounds when tested at neutral or alkaline reactions, and exhibit some susceptibility only in acid media (Baker, Harrison, and Miller, 1941; Scales and Kemp, 1941; Valko and DuBois, 1944). Among the antibiotic agents of biological origin which have been discovered recently, several exhibit striking selectivity in their action. Quinones isolated from molds or bacteria, as well as the synthetic products, are very active against streptococci, less against staphylococci, and practically inactive against colon bacilli (Armstrong, Spink, and Kahnke, 1943). Penicillin, gramicidin, actinomycin A, etc., are extremely active against many Gram-positive species, and little or not at all active against Gram-negative bacilli. Although gonococci and meningococci are susceptible to both penicillin and gramicidin, this exception is correlated with the fact that the pathogenic Gram-negative cocci occupy an intermediate position between the Gram-positive organisms and the Gram-negative bacilli (Dubos and Hotchkiss, 1941, 1942; Fleming, 1929; Waksman and Woodruff, 1942).

In contrast with this impressive array of agents selectively active against the Gram-positive forms, there are only few, if any, which are selective for the Gram-negative bacilli. The arsenites, the azides, and the tellurites, although active in very low concentrations against coliform bacilli, are inert toward some, but not all, Gram-positive organisms, in particular, pyogenic cocci. The addition of 1:100,000 sodium azide to sugar broth, for instance, is sufficient to inhibit the growth of most Gram-negative

organisms and permits the direct detection of streptococci in milk. Similarly, high dilutions of tellurites inhibit the growth on agar of the Gram-negative flora of the throat, whereas diphtheria and diphtheroid bacilli grow in media containing up to 0.1 per cent of these salts (Browning, 1930; Fleming, 1929; Gohar, 1941; Hartmann, 1936; Packer, 1943; Snyder and Lichstein, 1940). Some slow oxidizing agents (hypochlorites, dichromates, permanganates, ferricyanides, etc.) are claimed to be especially active against Gram-negative bacilli (Mallmann, Botwright, and Churchill, 1941). Finally, it may be mentioned that it is only in the case of these organisms that the phenomenon of immune bacteriolysis, which involves the participation of complement and of a specific antibody, has been recognized.

In order to account for these extraordinary differences, it might be conceived that the Gram stain divides the microbial world into two large groups, characterized by different metabolic systems which respond differentially to the varied inhibitors. It is very likely, for example, that the inhibitory effect of sodium azide on the growth of coliform bacilli is due to the fact that this substance inhibits aerobic metabolism. On the contrary, pneumococci, streptococci, lactobacilli and anaerobes which derive most of their energy from lactic acid fermentation can grow in the presence of the azide which does not interfere with this source of energy (Lichstein and Soule, 1944). In general, however, the fact that different bacterial species belonging to profoundly different metabolic types react identically to the Gram stain renders difficult any correlation between susceptibility to antiseptics and metabolic behavior. Furthermore, differences in staining properties are quantitative rather than qualitative in nature; Gram-positive and Gram-negative species form a more or less continuous series rather than two qualitatively unrelated groups. Finally, the many varied types of inhibitors which we have considered differ, not only in chemical nature, but also with reference to the physiological systems which they affect, and it appears likely that the factors which condition the selective susceptibility of Gram-positive and Gram-negative species reside, not in

qualitative differences of metabolic behavior, but rather in quantitative differences in physicochemical properties and cellular structure. Among these will be mentioned the acid base properties of the bacterial cells and their phospholipid constituents.

Relation Between the Acid Base Properties of the Cell and Its Susceptibility to Toxic Agents.—Analysis of the mode of action of the basic dyes has led to the formulation of a theory which accounts for the differential susceptibility of the Gram-positive and Gram-negative species to varied antibacterial agents. The bacteriostatic effect of gentian violet is illustrated in the following experiment. Graded concentrations of the dye were added to agar media; cultures of ten different bacterial species, five Gram-positive and five Gram-negative, were then streaked on these media in order to determine the minimal concentration of the dye sufficient to inhibit growth. The "gentian violet coefficients" of the different strains defined as the maximal dilution of inhibitor still capable of exerting its antibacterial effect on that strain is given in Table 41 (Churchman and Siegel, 1928).

TABLE 41

GENTIAN VIOLET COEFFICIENTS OF DIFFERENT BACTERIAL STRAINS

<i>Corynebacterium diphtheriae</i>	800 000	<i>Shigella dysenteriae</i> (Shiga)	40 000
<i>Bacillus anthracis</i>	650 000	<i>Eberthella typhosa</i> appr.	1 000
<i>Bacillus subtilis</i>	600 000	<i>Escherichia coli</i>	"
<i>Staphylococcus aureus</i>	500 000	<i>Proteus vulgaris</i>	"
<i>Rhodococcus roseus</i>	450 000	<i>Bacillus pyocyaneus</i>	"

Data from Churchman and Siegel (1928, chart 1, p. 76)

Although the composition of the medium, its pH, the metabolic activity of the strain under consideration, etc., greatly modify the value of the "gentian violet coefficient," it is clear that Gram-positive bacilli, either sporulated or nonsporulated, are in general much more susceptible to the dye than Gram-negative species. Unfortunately, no Gram-negative cocci are included in Table 41, but general experience indicates that these organisms are more resistant than the pneumococci and streptococci and somewhat more susceptible than the typical coliform bacilli.

It is now necessary to consider the fact that basic dyes are commonly used in the preparation of the selective media devised for the isolation of salmonella organisms from fecal material rich in other nonpathogenic Gram-negative bacilli. In other words, basic dyes can, in the proper concentration, inhibit the growth of some of the Gram-negative species and allow the growth of others. For example, brilliant green, added in a concentration of 1:150,000 to fecal suspension in broth, inhibits *Escherichia coli*, but permits the growth of *Eberthella typhosa*. This dye, which has been empirically selected in the preparation of the selective media, is the most basic of the triphenylmethane series, and, therefore, according to the view presented earlier in this discussion, is the most likely to react with cells exhibiting a high "iso-electric point." In fact, brilliant green, more basic than gentian violet, is also more efficient in inhibiting *E. coli* in high dilutions. Similarly, in the acridine series, the more basic members of the group are not only more active against Gram-positive, but also exhibit bacteriostatic activity against certain Gram-negative organisms (Albert, 1942).

It appears then that when used in low concentrations, the basic dyes inhibit the Gram-positive species while allowing the growth of Gram-negative bacilli. When used in somewhat higher concentrations, the most basic dyes also inhibit some of the Gram-negative organisms (*E. coli*), but allow the growth of others (*E. typhosa*). It is possible that these two sets of phenomena can be interpreted in terms of one and the same theory.

As already mentioned, the greater susceptibility of the Gram-positive species seems to be due to the fact that their cellular material is very acidic, and therefore exhibits great affinity for the basic dyes. The methods used for the determination of the overall iso-electric points of bacteria (Chapter III:2), while sufficient to differentiate between the typical Gram-positive and Gram-negative species, are too crude to allow a satisfactory classification of the Gram-negative bacilli according to the relative acidity of their cell constituents. Nevertheless, in one set of experiments where an attempt was made to measure the comparative affinity

of one strain of staphylococcus, four strains of *E. coli* and one strain of *E. typhosa*, for acid fuchsin and methylene blue at different pH, it was found that the staphylococcus behaved as the most acidic, and the typhoid bacillus as the least acidic structure; the different strains of *E. coli* varied in acidity from one to another, but all were more acidic than *E. typhosa* (Tolstoubov, 1929). If this difference between the colon and the typhoid bacillus is a significant and constant one, it may provide an explanation for the greater susceptibility of the former organism to basic dyes in general, and to brilliant green in particular. Extension of these observations to a number of different inhibitors, not only dyes but other cationic antiseptics, might serve as a basis for a rational method of preparation of selective media capable of differentiating between the different species within a given bacterial group.

It must be emphasized again that the expression iso-electric point as used in the present discussion expresses the H^+ concentration at which the cell material exhibits equal retention of anion and cation. This character certainly does not refer to a single component of the cell, but is probably an overall value for a number of independent cellular constituents. Moreover, the conditions under which the determination is made involve in most cases the death of the cell, so that the results are not applicable to the living organism. Nevertheless, it appears likely that the susceptibility of a given cell to a cationic antiseptic is conditioned in part by the acidity of the cell constituents and the basicity of the antiseptic agent. Although all bacteria are predominantly acidic, there is a notably different distribution of this quality among the different species. At one extreme are the Gram-positive organisms which have a high ratio of acidic to basic radicals, while the Gram-negative bacilli are at the other extreme. Marked differences exist within each group, intermediate types are known, and the two classes in reality form a continuous series. Gram-positiveness is therefore a qualitative property which is correlated in some obscure way with increased affinity for cationic dyes or other cationic antiseptics; the high basicity required for anti-

septic action becomes of increased importance in dealing with the Gram-negative species.

Protective Effect of Phospholipids Against Certain Toxic Agents.—Gram-negative bacilli and cocci, treated with reagents as varied as trichloroacetic acid, urea, diethylene glycol, phenol, trypsin, etc., yield in solution a molecular complex consisting of a phospholipid, a protein, and a polysaccharide (Chapter IV:2). The phospholipid, in the few cases where it has been studied, appears to be of the cephalin type (Morgan and Partridge, 1940). Similar complexes have not been recovered from the Gram-positive organisms, a fact which indicates that, in the Gram-positive cell, the distribution and organization of phospholipids, proteins, and polysaccharides does not permit them to be released as a soluble complex by the reagents mentioned above. Indirect evidence, especially of immunological nature, also suggests that this complex material extracted from Gram-negative bacilli is located at the cellular surface (Chapter IV:2). Moreover, phospholipids are capable of inhibiting a great many toxic agents, and, for instance, the presence of minute amounts of cephalin completely protects Gram-positive bacteria from the effect of gramicidin, both *in vitro* and *in vivo*. It is worth considering, therefore, that the phospholipid-protein-polysaccharide complex of the Gram-negative bacilli may contribute to the greater resistance of these organisms to many antiseptics.

Since basic substances such as histones or protamines are known to precipitate cephalins, an attempt has been made to determine whether they could also render Gram-negative bacilli susceptible to certain substances to which these organisms are otherwise resistant. It has been found, in fact, that whereas neither Tergitol-7 (a typical anionic detergent) nor tyrothricin have any effect when used alone on the respiration of *E. coli*, the addition to the system of protamine sulfate under conditions where this basic protein is itself inactive, brings about complete inhibition of bacterial respiration within five minutes. Preliminary experiments have indicated that other basic substances such as methylene blue and acridines have the same potentiating effect

as the protamine (Miller, Abrams, Dorfman, and Klein, 1942; Pittman, 1944) (fig. 32).

Thus, certain lipids, in particular the very acidic cephalins, protect Gram-positive organisms against the action of several toxic agents. Conversely, basic substances can render Gram-negative bacilli susceptible to antiseptics to which they would be resistant otherwise. Together with the evidence derived from the selective action of dyes on different bacterial groups, these facts

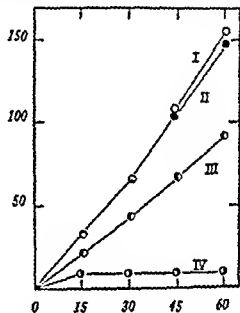


FIG. 32.—Effect of protamine and tyrothricin on respiration of *E. coli*. T. 38° C; atmosphere, air; pH 5.3; 5×10^9 cells per vessel. I. Control respiration. II. Tyrothricin (1:15,000). III. Protamine (1:3,000). IV. Mixture of tyrothricin (1:15,000) and protamine (1:3,000). (From Miller, Abrams, Dorfman, and Klein, 1942, fig. 1, p. 429.)

suggest that the surface properties of the cell are factors of great importance in determining the ability of the various types of toxic substances to reach the areas where they can interfere with cellular metabolism.

It is obvious that we have considered only a few of the very many factors which affect the susceptibility of a cell to a given inhibitor. Moreover, the ready production by most microbial species of variant forms differing from the parent strain by a greater

resistance to certain compounds, gives rise to many exceptions which obscure general laws. There is, however, no doubt of the existence of a correlation between behavior toward the Gram stain and resistance to injurious agents. The fact that it has been much easier to discover effective antiseptics for the Gram-positive organisms than for the Gram-negative bacilli, indicates that this correlation is not only of academic interest, but also of significance in the practical problems of chemotherapy.

4. CELLULAR STRUCTURES AND FUNCTIONS AFFECTED BY ANTIBACTERIAL AGENTS

Death Rate of Bacterial Cultures Subjected to Various Injurious Agents and Procedures.—The cells do not all die at once when a bacterial culture is subjected to heat or treated with a chemical disinfectant. The largest number die in the first time interval and the number killed in each period is a constant proportion of the numbers alive at the beginning of that period. In other words, the curve describing death rate in a bacterial population is similar to the curve expressing the rate of a first order reaction in which the reaction rate is determined by only one of the components of the system. The death rate in bacteria is therefore very different from that which is observed in higher organisms, in which case the death rate corresponds to a normal population distribution curve of resistance to the injurious agent under consideration. In order to account for the first order character of the death curve exhibited by bacterial cultures, it has been suggested that the single component of the system assumed to be responsible for the order of death might be a gene and that this gene might consist of a single molecule. It was also noted that the curve of sterilization of bacterial cultures not only presents great similarity with the curves of coagulation of proteins in pure solution, but also exhibits a very high temperature coefficient. These observations also agreed with the view that the first order character of the death curve is due to the fact that the lethal agent exerts its effect upon one particular protein in the organ-

ism (Chick, 1908, 1910, 1912, 1930; Rahn, 1929, 1932, 1934).

Bactericidal Effects of Radiations.—Analysis of the killing effect of radiations on bacteria has led to a number of conflicting interpretations. Some observers have claimed that death can be the result of absorption of a single x-ray quantum of energy. Since, according to the findings, only one in twenty of the absorbed quanta affects the susceptible cell constituents, the destruction of which leads to cell death, this constituent should have a volume less than 0.06 of the cell itself (Wyckoff, 1930). With ultraviolet light, four million quanta of energy are required to kill a single coliform bacillus, showing that death is not due, as it appears to be with cathode rays, to a single quantum absorption, but to a more generalized effect on the bacterial population (Wyckoff, 1932). The validity of the "single photon hit" theory has been questioned by many investigators (Hollaender, 1942; Lea and Haines, 1940; Lea, Haines, and Bretscher, 1941; Lea, Haines, and Coulson, 1936; Luria, 1939; Rentschler and Nagy, 1942). In any event, it appears that the most effective wave length in the ultraviolet range is the region 2650 Å, close to the wave length at which nucleic acids are most highly absorbent; only a few biologically active compounds (riboflavin) show definite absorption bands in the longer wave lengths radiation which exhibit a much less effective bactericidal effect (Dreyer and Campbell-Renton, 1936; Hollaender, 1943). Gamma and x radiations are claimed to exert their metabolic effects by causing an inhibition of the synthesis of nucleic acid in the nucleus and an accumulation of ribonucleotides in the cytoplasm. This change might be due to the failure of ribonucleic acid to be reduced to the desoxyriboform in the nucleus, resulting in an inhibition of mitosis (Mitchell, 1942, 1943). It may be mentioned at this time that the lethal action of radiations has also been considered to be due to the production of lethal mutants (Lea, Haines, and Bretscher, 1941). Finally, many workers have observed that, under the influence of radium and ultraviolet light, cellular division is interrupted although elongation may proceed, indicating that growth and division behave as two independent functions which exhibit differen-

tial susceptibility to radiations (Bouchard and Balthazard, 1906; Bruynoghe and Mund, 1925; Lea, Haines, and Coulson, 1937).

Differential Susceptibilities of the Various Metabolic Systems.—With increased knowledge of the chemistry of cellular structure and metabolism, more and more attempts have been made to analyze the phenomena of bacteriostasis and bactericidal effect, not by the study of overall death curves, but rather in terms of the chemical reactions which take place between the different agents and the cellular structures and functions which they affect. It must be emphasized, however, that the discovery that a given antiseptic inactivates a certain enzyme or cellular constituent does not constitute proof that this particular reaction is the one which determines the bacteriostatic or bactericidal effect. There are few agents, if any, which are so specific in their action that they affect only one cellular function or structure. Even the most highly purified preparations of penicillin, for example, are reputed to inhibit the enzyme urease, although it is most unlikely that the antibacterial effect depends upon this inhibition (Turner, Heath, and Magasanik, 1943). Similarly, sulfanilamide inhibits carbonic anhydrase, a fact unrelated to the mechanism of its action against bacteria (Mann and Keilin, 1940). The fact that the different functions of a given cell are inactivated at different rates by a given agent accounts for the observations that there exists no correlation between bactericidal effect and inhibition of metabolic enzymes when bacteria are treated by silver and other germicides, or by ultraviolet radiation (Braun and Vasarheliji, 1940; Bucca, 1943; Cook and Stephenson, 1928; Lodge and Hinshelwood, 1943; Spray and Lodge, 1943; Yudkin, 1937b). As will be emphasized later, most of the studies of the mechanism of action of antiseptics on bacteria have been concerned exclusively with the inhibition of catabolic reactions. It is possible, however, that the phenomena of synthesis and of cell division are in many cases more susceptible, and are the first ones to be affected, while the enzymes concerned in oxidoreduction become inhibited only at higher concentrations of the antibacterial agents.

The Destructive Effect of Certain Enzymes and Surface Active Substances on Cellular Structures.—In only a few cases has it been possible to ascribe the death of bacteria to the direct destruction of their cellular structure by the killing agent. The enzyme lysozyme, for instance, probably causes the death and lysis of several bacterial species by hydrolyzing a mucopolysaccharide which is an essential constituent of the framework of the susceptible cells (Chapter IV:1). It is rather surprising that other enzymes capable of attacking other exposed structural constituents of bacteria have not been described, and it seems that search for such enzymes would offer an interesting field of investigation. In this respect, it is worth pointing out that immune bacteriolysis, a phenomenon which depends upon the joint action of specific antibody and active complement on living cells, probably involves the participation of some enzyme responsible for the actual lysis.

Obvious destruction of cellular structure can also result from the action of certain detergents. The antibacterial and lytic activity of these surface active substances does not vary in proportion to their ability to depress surface tension, but depends upon some structural properties of the molecule. Moreover, complete inhibition of metabolism and bactericidal effect can occur, without being accompanied by lysis, depending upon the type of bacteria and the nature of the detergent.

It has been suggested that the extremely rapid effect of synthetic detergents on bacterial metabolism and viability depends on a twofold action: first, a disorganization of the cell membrane by virtue of the great surface activity of these compounds; and second, a denaturation of certain proteins essential to metabolism and growth. Studies on model systems have revealed that detergent-like compounds which penetrate lipoprotein monolayers increase surface pressure markedly and cause lysis, whereas compounds which do not penetrate but are adsorbed cause agglutination. It is conceivable that similar disorientations and alterations in surface forces occur in the bacterial cells (Baker, Harrison, and Miller, 1941). Thus, detergents in general, and

in particular the antibacterial basic polypeptide tyrocidine, so modify the cell surface that vital soluble metabolites such as organic nitrogen compounds, inorganic phosphate, and phosphate esters, are immediately washed out of the cell, a situation obviously incompatible with the maintenance of life. This phenomenon is observed, not only when the cells are lysed by the detergents or tyrocidine, but also in the case of bacteria which, like streptococci, do not show any obvious evidence of lysis (Hotchkiss, 1944).

Dissolution of pneumococci by surface active substances (as illustrated by bile "solubility") is only a secondary phenomenon, dependent upon the activity of the autolytic enzymes. The primary injury caused by bile or other detergents is probably the destruction of the plasma membrane. This effect is associated with the inactivation of the metabolic systems upon which depends the maintenance of organized cell structure and brings into contact enzymes and substrates which are separated in the intact living cell (Chapter IV:3). In addition to the fact that they can peptize cellular structures, detergents are also effective protein-denaturing agents, and dissociate conjugated proteins (Anson, 1939; Kubn and Biellig, 1940). Their activity in high dilutions depends probably upon the fact that, on account of their surface activity, they can concentrate at interfaces.

In the light of this knowledge it is not surprising that different phospholipids, added to the bacterial suspension at the same time as or before the detergents, completely inhibit the toxic effects of the latter. Like detergents, phospholipids possess a characteristic polar-nonpolar structure, and presumably exhibit a similar affinity for the bacterial cells. Since they do not by themselves affect bacterial metabolism or viability, they probably protect the cell by altering the structure of the membrane so as to prevent penetration of the toxic agents (Baker, Harrison, and Miller, 1941).

Inhibition of Metabolism by Detergents and Other Antiseptics.—Many attempts have been made to identify the cellular reactions interfered with by the different antiseptics and chemo-

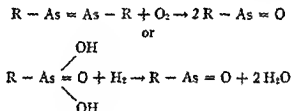
therapeutic agents. Thus, dilution of 1:3,000 to 1:30,000 of many cationic detergents immediately and irreversibly inhibit oxygen uptake and acid production of all bacterial species tested, whereas the metabolism of Gram-negative bacilli is unaffected by anionic detergents under the same conditions (Baker, Harrison, and Miller, 1941). Although there exists in general a definite parallelism between bactericidal effect and inhibition of oxidoreduction, the nature of the initial injury is not revealed thereby. It is probable that the effect of detergents is not limited to interference with one single enzyme but depends on a nonspecific effect on cell membranes and proteins.

Bacterial cells treated with phenolic antiseptics exhibit a decrease of respiration before killing effects can be observed, and certain phenols and aliphatic alcohols inactivate succinic dehydrogenase. The hydroxy derivatives of phenothiazine react with heme pigments and in particular can reduce cytochrome C irreversibly (Collier and Allen, 1941; Sykes, 1939). Since the antiseptic phenols are surface active agents, and since the phenolic hydroxyl group can react with the amino group of proteins, especially on surfaces, it is very likely that, in this case again, the phenols do exert their antibacterial activity through a destructive action on several important constituents and structures of the cell.

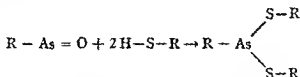
Effect of Dyes on Cellular Metabolism.—Little is known of the nature of the cellular constituents affected by the dyes. It has long been claimed that Janus green inhibits proteolytic enzymes; pyocyanine has been shown to inhibit succinic dehydrogenase (Keilin and Hartree, 1940); some of the dyes can act by maintaining the redox potential of the bacterial environment above a figure compatible with metabolic activities and bacterial growth (Dubos, 1929; Fildes, 1929; Hoffmann and Rahn, 1944). We have already described the reaction between the basic dyes and various groups of the cell, among which can be mentioned the —COOH radicals, and the more strongly acid phosphoric groups which are particularly important in the nuclear apparatus and in some of the coenzyme systems (Chapters III:2 and VIII:1).

It is of special interest in this respect that some of the growth inhibiting effect of acriflavine can be neutralized by the addition to the medium of nucleotides. Since these substances form complex salts with acriflavine, the dye may inactivate enzyme systems of which nucleotides are an essential part, although it is also possible that the added nucleotides act by protecting susceptible cellular structures against the toxic action of the dye (McIlwain, 1942h).

Effect of Arsenicals and Mercurials on Cellular Metabolism.—It was suggested very early that the affinity of arsenicals for reduced sulfur might account for the toxic action of these compounds (Ehrlich, 1909a, b). More specifically, it is now considered that the arsenicals, whether trivalent or quinquevalent, are first oxidized or reduced respectively to the corresponding arsenoxides:



the arsenoxides in turn reacting with some reduced sulfhydryl groups of the cell



It is pointed out in support of this view that the injection simultaneously with a trypanocidal drug of compounds containing an —SH group (reduced glutathione, thioglycolic and thiolactic acid, or cysteine) slows the rate of disappearance of trypanosomes from an infected animal. Interpretation of the mode of action of arsenicals is rendered more complex by the fact that neoarsphenamine

is much more rapidly lethal to trypanosomes than to spirochetes. When a mixture of the two organisms is treated with different concentrations of neoarsphenamine, the trypanosomes are killed in two hours by a dilution of 1 in 10,000,000, whereas the spirochetes remain healthy for much longer in higher concentrations of the drug and are killed only after twenty-four hours by a dilution of 1 in 500,000. To account for this difference, it has been suggested that neoarsphenamine can be absorbed by trypanosomes in the unchanged condition, whereas oxidation to the arsenoxide is necessary before it can enter the spirochetes (Findlay, 1939, page 277; Simic, 1923). In the case of the trypanosomes, the neoarsphenamine is rapidly taken up and becomes oxidized to the arylarsenious oxide either on the surface or within the cell, by the normal cellular oxidation processes. In the case of the spirochetes, either the rate of absorption, or the rate of oxidation to the arylarsenious oxide, is a much slower process (Findlay, 1939, page 277; Papamarku, 1927).

Although the antiseptic activity of the mercuric ion had generally been ascribed to its ability to precipitate proteins, Hg^{++} , like arsenoxide, possesses a great affinity for sulfhydryl groups (Fildes, 1940b). In fact, the antibacterial effect of mercury can be specifically neutralized by the addition of $-SH$ compounds to the system, and can even be reversed after prolonged exposure of the bacteria to the antiseptic by the addition of reduced sulfur compounds which combine with the metal (Chapter VIII:2). The hypothesis of the antisulfhydryl action of Hg^{++} accounts for some of the observations which are incompatible with the view that mercurial germicides act only by virtue of their ability to precipitate proteins, such as the fact that, although organic mercurials are even less highly ionized than inorganic salts, or not at all, they are often more active against bacteria.

Granted that arsenicals and mercurials owe their biological activity to their affinity for the sulfhydryl group, the nature of their action is not thereby clearly defined. It has often been assumed that glutathione is the sulfur compound of the cell involved in the toxic effect. Since this substance is the coenzyme of glyoxalase,

adequate concentrations of the antiseptics would inhibit the activity of the enzyme. It must be remembered, however, that, in living tissues, the —SH group occurs not only in glutathione, but also in a number of other constituents, particularly in enzyme proteins, such as urease, triosedehydrogenase, the cathepsin group of proteases, etc.; many of these enzymes become inactive upon oxidation of the sulfhydryl group.

The carbohydrate metabolism of many pathogenic trypanosomes susceptible to arsenic is so intense that in twenty-four hours a trypanosome consumes twice its own weight of sugar. *T. cruzi* which does not possess this intense activity is not acted upon by arsenicals, while the nonpathogenic *T. lewisi*, which is also relatively resistant, consumes only a fifth of the sugar required by pathogenic, arsenic-susceptible trypanosomes (Findlay, 1939, page 225). These facts appear to suggest an effect of arsenicals on carbohydrate metabolism. On the other hand, arsenic can combine with the iron of respiratory pigments but it also exerts a toxic action on liver lipase and other enzymes (Clark, 1937). In addition to its affinity for —SH, Hg^{++} can combine with other active chemical radicals of the cell, such as free amino groups (Salle and Ginoza, 1943). That the influence of inorganic mercury on susceptible cells is not yet fully understood appears from the fact that the oxygen consumption of yeast is completely inhibited by this poison when glucose is the substrate, whereas it is unaffected when lactic acid or pyruvic acid is used or when the cell is in a state of endogenous metabolism (Selzer and Baumberger, 1942). Although there is no doubt that the reaction with sulfhydryl groups accounts for much of the biological activity of mercurials and arsenicals, it is probable, therefore, that more than one enzyme system can be affected by these agents and that much remains to be learned of the metabolic disturbances which they cause (Eagle and Mendelsobn, 1938; Findlay, 1939, pages 224–277).

The Production of Nutritional Deficiency by Sulfonamides.—Attempts to explain antibacterial action in terms of specific inhibition of some essential metabolic steps have received their chief

impetus from the discovery that p-aminobenzoic acid can neutralize the bacteriostatic action of sulfonamides. It was assumed that PABA is an essential metabolite for bacteria (a fact now established experimentally), and that the drug, by virtue of similarity in chemical structure, can compete with it in some essential metabolic reaction (Woods, 1940) (Chapter VIII:1).

A number of other analogous types of inhibition caused by substances capable of competing with essential metabolites have since been described. Pyridine-3-sulfonic acid and its amide can inhibit bacterial growth by competing with nicotinamide. The aminosulfonic acid homologues of natural carboxy amino acids also inhibit bacterial growth and the inhibition is decreased or removed by the addition to the medium of amino acids. Moreover, when staphylococci are made independent of most added aminocarboxylic acids by training, the α -aminosulfonic acids are no longer inhibitory (McIlwain, 1941a, b). Inhibition of growth by pantooyltaurine (N- α -dihydroxy $\beta\beta'$ -dimethyl butyryl taurine) can be reversed by the addition to the medium of pantothenic acid, the essential growth factor related to the inhibitor. Organisms which synthesize pantothenic acid are not inhibited by the sulfonic acid compound (Kuhn, Wieland, and Möller, 1941; Snell, 1941). Inhibition of growth by the pyridine analogue of thiamine (pyrithiamine) is observed only in those bacteria which require thiamine (or its components pyrimidine and thiazole) for growth; the inhibition is overcome by sufficient amounts of the vitamin (Woolley and White, 1943; Wyss, 1943). Similarly, desthiobiotin exerts an antibiotin effect for *Lactobacillus casei* (Dittmer, Melville, and du Vigneaud, 1944; du Vigneaud, Melville, *et al.*, 1942; Lilly and Leonian, 1944). In all these cases it is likely that bacteriostasis is due to the blocking of reactions essential to growth by inhibitors which act on account of their structural similarity to the normal metabolite or growth factor involved. It is possible, therefore, that a rational approach to the production of new chemotherapeutic agents will consist in modifying the structure of essential metabolites so as to produce

substances which can no longer exhibit any specific action in the cell economy, but which are still able to block the enzymes concerned in the reaction (Fildes, 1940a; McIntosh and Whitby, 1939) (fig. 33).

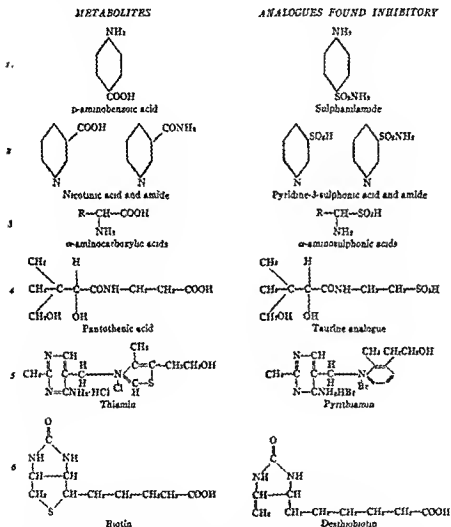


FIG. 33.—Applications of structural analogy with metabolites to the synthesis of inhibitors.

1-4. (From Mellin, 1942a, p. 412.)

5. From Robbins, 1941, p. 419 and Wooley and White, 1943.

6. (From Du Vigneaud, Melville, *et al.*, 1942, p. 475).

Although the general principle of competitive inhibition has thus been established, the precise nature of the inhibitory reactions has not yet been elucidated, not even in the case of the sulfonamides (Henry, 1943). It would fall outside the scope of the present discussion to attempt to evaluate the many views expressed to account for it; to decide, for instance, whether the primary effect of the sulfonamides is to block the respiratory processes of susceptible organisms and thus cause inhibition of growth (Sevag and Sbelburne, 1942), or whether the drugs prevent the synthesis of some essential metabolite or compete with it for a given enzymic system and affect respiration only indirectly (Van Niel, 1943b; Wyss, Strandkov, and Schmelkes, 1942). In any event, it is clear that, unlike the detergents, the phenols and many other types of general protoplasmic poisons, the sulfonamides do not cause a complete inhibition of oxygen uptake and acid production. It would be of great interest to test the effect on the metabolism of susceptible cells of pyridine-3-sulfonic acid, α -aminosulfonic acids, pantoyl taurine, etc., compounds which bear a similar relation to the essential metabolites, nicotinic acid, aminocarboxylic acids, and pantothenic acid, as sulfanilamide does to p-aminobenzoic acid. If these inhibitors should affect the oxygen uptake and acid production of susceptible cells, it would indicate the possibility that an agent can inhibit the respiratory systems by an indirect route, although its primary effect on cell growth results from the inhibition of some other cellular reaction. As already stated, the effect of these analogues of essential metabolites can be interpreted as causing a state of nutritional deficiency, and can be neutralized by the addition to the medium of an excess of the appropriate metabolite, or by the production by the organism under consideration of adequate amounts of these same substances (McIlwain, 1942a, 1943a, b). Certain organisms relatively insusceptible to sulfonamides have been shown, for instance, to produce during normal growth p-aminobenzoic acid in excess of their needs and in quantities with which the sulfonamide cannot compete (Landy, *et al.*, 1943). *E. coli*, which is known to synthesize excess of nicotinamide derivatives, is not affected

by inhibitors which interfere with nicotinamide metabolism, while *Staph. aureus*, *Proteus vulgaris*, and *Strept. hemolyticus* which cannot perform all such syntheses, are inhibited. One given amino-sulfonic acid tested against different strains of *P. vulgaris* exhibits marked differences in inhibitory activity, probably because particular strains differ in synthesizing abilities or in need for the corresponding aminocarboxylic acid (McIlwain, 1942a).

The Significance of Anabolic Reactions in the Phenomena of Antibiosis.—The great majority of studies dealing with the mechanism of action of antibacterial agents emphasize their effect on catabolic reactions, measured in terms of oxygen uptake, acid production, dehydrogenase activity, etc. It appears possible, however, that, in some cases at least, the essential primary effect of the inhibitor is on a synthetic process, the effect on respiration and fermentation being only an indirect, secondary phenomenon. Unfortunately, so little is known of the synthetic activities of the cell that only few methods are available for the study of anabolic processes and of their possible relation to antibacterial action.

Certain growth inhibitors (azide, dinitrophenol) do not abolish catabolic reactions and, in fact, markedly stimulate oxygen uptake by yeast, *E. coli*, *Ps. saccharophila*, and certain algae. Although dinitrophenol can directly stimulate the rapid oxidation of assimilated material, it appears that this agent as well as the azides can also act indirectly by blocking synthetic reactions which result in the production of polysaccharides, and thus, by allowing the complete oxidation of the substrates, cause an apparent stimulation of metabolism. It is interesting in this respect that dinitrophenol has a marked inhibitory effect on phosphate uptake by yeast and that a number of antibacterial agents (sulfonamides, penicillin, gramicidin) inhibit bacterial growth without abolishing oxygen uptake and other catabolic activities (Clifton and Logan, 1939; Doudoroff, 1940; Hotchkiss, 1944; Pickett and Clifton, 1941, 1943; Van Niel, 1940, 1943b; Winzler, 1940, 1944).

Propamidine completely inhibits bacterial metabolism in the same concentration and under the same conditions which are effec-

tive in inhibiting bacterial growth, and it is very likely, therefore, that this drug exerts its antibacterial effect through a direct inhibition of the oxidative metabolism of susceptible bacteria. On the contrary, the sulfonamides do not abolish completely the respiratory activity of microorganisms, especially in the low concentrations which are sufficient to inhibit growth. The facts that there exists no parallelism between inhibition by these drugs of respiration and of growth, and that the respiration of a sulfonamide-sensitive and of a sulfonamide-resistant strain is equally inhibited, suggest that, in this case, inhibition of the catabolic reactions is not the primary phenomenon. Whether anabolic reactions such as the synthesis of methionine are the first ones to be affected remains an interesting but unproven possibility (Bernheim, 1943; Henry, 1943; Kohn, 1943b; Sevag and Shelburne, 1942; Van Niel, 1943b; Wyss, Strandskov, and Schmelkes, 1942).

When tested under the proper conditions, gramicidin markedly increases the rate of oxygen uptake and of acid production by pneumococci, streptococci, and staphylococci, even when used in concentrations far in excess of those sufficient to inhibit growth (Duhos, Hotchkiss, and Coburn, 1942). Washed normal staphylococci, respiring in glucose, steadily remove inorganic phosphates from the medium, but cannot do so when small amounts of gramicidin are present, although respiration is not prevented (Table 42) (Hotchkiss, 1944). Of course, many poisons, like HgCl_2 , halogen compounds, and most antiseptics inhibit phosphate uptake, but at the same time prevent metabolic activity. Assuming a polysaccharide synthesis analogous to that which takes place in animal tissues, one may suppose the existence in staphylococci of enzymes located at or near the cell surface, which are capable of utilizing external inorganic phosphate and introducing it as phosphorylated carbohydrate inside the cell. Blocking of this synthesis at some point by gramicidin would tend to limit (a) polysaccharide accumulation, (b) phosphate uptake, and (c) cell multiplication (Hotchkiss, 1944).

Unfortunately, no information has been published concerning

TABLE 42

EFFECT OF GRAMICIDIN ON OXYGEN AND PHOSPHATE UPTAKE BY STAPHYLOCOCCI RESPIRING IN GLUCOSE

MOLAR CON- CENTRATION OF PHOSPHATE	P PRESENT IN MEDIUM μg	WITHOUT GRAMICIDIN		WITH GRAMICIDIN	
		O ₂ uptake μl	P uptake μg	O ₂ uptake μl	P uptake μg
0	0	107	0	151	0
0.0009	41	105	41	163	0.5
0.0022	95	105	42	160	0
0.0049	214	109	41	165	0
0.0097	420	111	39	172	0
0.0288	1250	113	43	227	0

From Hotchkiss (1944, Table IX, p. 185).

the mode of action of penicillin, except for brief statements that the substance interferes with the dismutation of pyruvate by susceptible staphylococci (Welshimer, Krampitz, and Werkman, 1944). It has been reported and confirmed that penicillin does not completely interrupt protoplasmic growth, but prevents cell division with the frequent production of giant forms and subsequent lysis of the cells (Gardner, 1940; Smith and Hay, 1942; Weiss, 1943). It is interesting in this respect that much evidence has been obtained from other sources that growth and cell division are two independent functions and that treatment with certain antiseptics or certain radiations permits interruption of the latter without interfering with the former, and thus results in the production of giant, elongated forms (Bruynoghe and Mund, 1925; Foster and Woodruff, 1943; Lea, Haines, and Coulson, 1937; Lodge and Hinshelwood, 1943; Spray and Lodge, 1943).

The observations which have just been reported do not allow any positive statement concerning the mechanism of action of chemotherapeutic agents. They suggest, however, that, in certain cases, antibiosis may be the result, not of inhibition of respiratory and other catabolic reactions, but rather of inter-

ference with unidentified reactions concerned in synthesis or cell division.

5. MECHANISM OF DRUG FASTNESS

Occurrence of Drug Fastness.—When mice infected with *Trypanosoma brucei* are treated with the dye fuchsin, the parasites rapidly disappear from the blood stream. They reappear after a week or two and can again be eliminated by further treatment with the same drug. The process, however, cannot be repeated indefinitely; after a time the parasites no longer respond to the administration of fuchsin and are found to have become resistant to the dye both *in vitro* and *in vivo* (Ehrlich, 1907b). This initial observation was soon followed by the discovery that trypanosomes can be rendered resistant to a number of other toxic agents, particularly to the arsenicals. Since then, the general phenomenon of fastness has been recognized in many types of living cells; it occurs particularly readily in bacterial species with reference to every possible kind of antiseptics, and of other injurious agents and procedures. Although much has been learned concerning the mechanism of production of resistant variant strains in bacteria (Chapters V:4 and VIII:5), it is in the case of trypanosomes that the nature of the cellular modifications which result in drug fastness has been extensively investigated. We shall outline the information available concerning the phenomenon in these protozoa in the hope that it may serve as a guide for the study of its counterpart in bacteria.

The Fixation of Dyes and Arsenicals by Susceptible and Resistant Trypanosomes.—Susceptible trypanosomes readily take up para-fuchsin and are stained by it, whereas resistant variants of the same species remain unstained under the same conditions. Similarly, normal and resistant trypanosomes exhibit a strikingly different behavior toward vital staining with acridine dyes. The parabasal body which is destroyed when normal forms are exposed to acriflavine is not affected when resistant forms are used. Penetration of the dye and its fixation on the blepharoblast and cyto-

plasmic granules can be demonstrated by observation of the parasites in fluorescent light; the reaction is completed in a few minutes in the normal, but does not take place in the resistant forms. The evidence appears convincing, therefore, that trypanosomes resistant to aromatic compounds escape injury because they fail to absorb the drug, or perhaps more exactly because the drug fails to reach the susceptible cellular substrate (Ehrlich, 1909a; Fischl and Singer, 1935; Gonder, 1912; Hasskó, 1932; Hawking, 1937; Jadin, 1932; Leupold, 1925; Pedlow and Reiner, 1935; Röhl and Gulbransen, 1909; von Jancsó, 1931-1932).

This general mechanism of resistance applies not only to the case of the dyes, but also to other types of toxic substances. *In vitro*, normal susceptible trypanosomes remove from the medium appreciable quantities of the trivalent arsenicals, neoarsphenamine, sulpharsphenamine, stabilarsan, and 3-amino-4-hydroxyphenylarsenoxide, whereas resistant forms do not. Compounds to which the atoxyl resistant trypanosomes show no resistance, such as phenylarsenoxide, sodium arsenite, and tartar emetic, are absorbed to the same extent by normal and resistant organisms. Arsenophenylglycine, which is less active on the resistant trypanosomes than on the normal ones, is also absorbed in smaller amounts by the former (Hawking, 1937; Reiner, Leonard, and Chao, 1932; Yorke and Murgatroyd, 1930; Yorke, Murgatroyd, and Hawking, 1931).

Molecular Structure of the Drug and Specificity of Drug Fastness in Trypanosomes.—Strains of trypanosomes rendered resistant to one or another arsenical exhibit in their resistance a type of specificity which has not yet been fully elucidated. Thus, although a strain resistant to atoxyl (*p*-aminophenylarsonate) is also resistant to a great number of substitution derivatives of phenylarsonic acid, it is still susceptible to arsenophenylglycine and other arsenicals, such as arsenophenoxyacetic acid and arsenophenylthioglycollic acid, which contain the acetic acid radical. Ehrlich postulated the existence of acetico receptors in the trypanosome cell to account for this anomaly. However, it is possible to produce strains of trypanosomes which are resistant to arseno-

phenylglycine and these strains are also resistant to arsacetin. Of special importance was the discovery that atoxyl-resistant strains are also resistant to dyestuffs of the acridine, oxazine, thiazine, selenezine, and pyronine series, although they remain susceptible to trypan red or to the triphenylmethane dyes. As already mentioned, strains resistant to atoxyl remain practically unstained by oxazine dyes, whereas normal strains are rapidly stained vitally and die soon after. Since strains resistant to atoxyl are also resistant *in vitro* to a series of substituted phenylarsenicals, but not to sodium arsenite, the conclusion seems justified that resistance to atoxyl is directed not against arsenic, but rather against the substituted phenyl radical in the various aromatic arsenicals tested. An interesting exception is found in phenylglycine arsonic acid to which atoxyl resistant strains are as susceptible as normal strains, whereas they are not affected by other arsonic acids such as atoxyl, tryparsamide, and arsacetin. Strains made fast to atoxyl, arsacetin, tryparsamide, reduced tryparsamide, neoarsphenamine, or acriflavine, appear to be identical, and strains made resistant to arsenophenylglycine exhibit moderate resistance to other aromatic arsenicals (Ehrlich, 1909b, 1910; King and Strangeways, 1942; Morgenroth, 1914; Roehl, 1909; Yorke, 1932; Yorke, Murgatroyd, and Hawking, 1931).

Relation Between Drug Fastness and Cellular Permeability of Trypanosomes.—Ehrlich postulated that a loss or a decrease of affinity of the receptors specific for the drug occurs in the resistant cell. This theory has been used to explain a number of observations, often without much experimental evidence of the validity or even of the plausibility of the explanation. Thus, the fact that trypanosomes can be made resistant to many varied antiseptics, has been explained by assuming the existence in these protozoa of a very large number of receptors—arseno receptor, orthoaminophenol receptor, acetico receptor, receptors for acriflavine, for parafuchsin, for diamidine compounds, for Bayer 205, etc.—specific for each inhibitor.

The observation that arsenic-resistant trypanosomes remain susceptible to phenylarsenoxide but not to substituted phenyl-

arsenoxides, has been interpreted as due to some intense affinity of the former compounds for the receptors; there is in reality no evidence that phenylarsenoxide possesses a greater chemical reactivity than the substituted derivatives, but it is possible that substitution in the molecule can modify its specificity and its ability to react with certain components of the susceptible cells. The receptor theory has also been used to account for the differential susceptibility of tissue cells and parasites for the arsenicals. Certain side chains ($-\text{NH}_2-\text{OH}$), present in the arsenicals effective as chemotherapeutic agents, are assumed to prevent these compounds from becoming fixed on animal cells, without affecting their affinity for normal trypanosomes. On the other hand, the receptors of the resistant trypanosomes are considered to be so modified that they now behave like those of animal cells (Hawking, 1937; King and Strangeways, 1942).

Analysis of the relations between the chemical structure of various trivalent arsenical compounds and their action *in vitro* upon normal and tryparsamide-resistant trypanosomes (*T. rhodiense*) may open the way for a somewhat simpler interpretation of some of the complex and varied phenomena of drug fastness. According to this study, arsenicals can be divided into three classes:

(1) Those which contain solubilizing carboxyl groups and form neutral sodium salts possessing a great affinity for water, e.g. 4-carboxy phenyl arsenoxide. These compounds are of low toxicity to normal as well as resistant trypanosomes (lethal dilution 1:800,000); it is considered that they enter the trypanosomes in the same way as glucose and salts, and that their low activity is due to their great solubility. Since this class includes the arsenoxide corresponding to arsenophenyl glycine, it is not necessary to postulate the existence of special "aceto-receptors" to account for the abnormal behavior of this compound.

(2) Those which are poorly soluble because of the absence of hydrophilic groups, apart from the arsenoxide group, e.g. phenyl arsenoxide and xylylarsenoxide. They are very actively trypanocidal for both normal and resistant trypanosomes (lethal dilu-

tion 1:640,000,000). It is believed that these substances are taken up at some lipid and water interface and are thus transported to the site where the arsenoxide can exert its lethal action.

(3) Those which act on resistant trypanosomes much less readily than on the normal forms, e.g. thioarsenic derivatives of trypanamide arsenoxide (lethal dilution 1:13,000,000 for normal and 1:400,000 for resistant trypanosomes). These compounds are taken up by the same groups in the parasite as those which take up oxazine and acridine dyes. The nature of the cellular structure involved is not known; it is probably of polar nature and is incapable of absorbing dyes of the triphenylmethane and Congo red type. It appears that both ends of the arsenical molecule are involved in the primary fixation, since phenyl-pp'-diarsenoxide ($\text{O}=\text{As} \text{---} \text{As}=\text{O}$) belongs to this third class and not to the second class (King, 1943; King and Strangeways, 1942) (Table 43).

The three different methods which have just been discussed, and by which the arsenicals become attached to the trypanosome, concern only the primary phase of drug action, i.e. its fixation on, or in the cell. The final and effective stage of trypanocidal effect is of a different order and concerns the action of the chemically reactive arsenoxide group, the toxophoric group, on some essential cell constituents upon which the life of the cell depends, probably a reduced sulfhydryl group (Chapter VIII:4). The possibility remains therefore that, in addition to the type of fastness which results from failure of the arsenoxide to reach its susceptible substrate, there may be another type depending upon some change, qualitative or quantitative, in the metabolism of the cell, which expresses itself in a greater ability to withstand the action of the toxophoric group proper.

Role of Selection in the Development of Drug Fastness in Bacteria.—The ease with which bacteria can be grown and enumerated in artificial media has permitted a study of the origin of the resistant forms issued from susceptible strains. Attempts have been made in particular to establish whether the resistant

TABLE 43

THE EFFECT OF MOLECULAR STRUCTURE ON THE DEVELOPMENT OF RESISTANCE TO ARSENICALS

COMPOUND	DILUTIONS IN MILLIONS LETHAL IN 6 HOURS		
	Normal Strain	Resistant Strain	N/R Ratio
Phenylarsenoxide, Ph AsO	320	640	0.5
Diglutathionylphenylthioarsinite, Ph As SG	80	80	1
p-Xylarsenoxide, $\text{Me}_2\text{Ph AsO}$ SG	205 160 205 80	205 320 205 160	0.5-1 0.5-1
p-Methoxyphenylarsenoxide, MeO Ph AsO	32	1	32
Phenyl'-pp-diarsenoxide, AsO Ph AsO	51.2	1.6	32
p-Acetanilidearsenoxide, NHAc Ph AsO			
Diglutathionyltryparsamidethioarsenite SG	12.8	0.4	32
$\text{CONH}_2\text{CH}_2\text{NHPhAs}$ SG	51.2 51.2	0.8 0.8	64
Benzamide-p-arsenoxide, $\text{CONH}_2\text{Ph AsO}$			

Data from King and Strangeways (1942, Table 11, p. 50)

bacteria always occur in small numbers during normal growth in the presence of the drug, or whether they are produced only as a response to the presence in the medium of the substance with reference to which resistance develops (Chapter V:4).

Evidence for the former point of view is found in a study of the survival and growth of pneumococci on solid media containing sulfathiazole, the number of pneumococci and the concentration of sulfathiazole per unit volume of medium being varied independently. In the parent susceptible strain, a very small but constant proportion of the total number of pneumococci are capable of growing in the presence of sulfathiazole, this propor-

tion being inversely related to the concentration of the drug. Secondary cultures derived from individual colonies grown once in sulfathiazole contain much higher proportions of cells capable of growing in the presence of the drug, and these proportions are directly related to the concentrations in which the first growth had occurred. This increased capacity remains constant through numerous subcultures in drug free media (Horsfall, 1942). These observations indicate that, during growth in the presence of sulfathiazole, there occurs a selection of the few individuals endowed with higher resistance to the drug, which are normally present in any susceptible culture. On the other hand, the fact that the most resistant organisms normally present in sensitive parent strains are significantly less resistant to sulfapyridine than the organisms of the corresponding resistant strains has led other investigators to feel that the most highly resistant individuals are formed as a result of some action of the drug on the sensitive organisms (Schmidt and Sesler, 1943). This conclusion involving as it does a belief in a transmissible effect of the environment on the germ plasm would require much additional evidence before being acceptable. It appears more likely that drug fastness is the result of progressive selection of the more resistant individuals occurring normally in any given population. Similar observations, liable to the same interpretation, can readily be made with reference to the production of variant forms of pneumococci, streptococci, and especially staphylococci resistant to penicillin and gramicidin.

Convincing evidence of the role of selection in the development of drug fastness in bacteria has come from the analysis of the comparative nutritional requirements of susceptible and resistant forms. It is possible to "train" staphylococci and diphtheria bacilli to grow in the presence of α -aminosulfonic acids or pantoyletaurine without ever exposing the organisms to either of these drugs, by training them to grow in the absence of α -aminocarboxylic acids or of pantothenic acid. The decrease in nutritional requirement is paralleled by an increased resistance to the inhibitor, and both changes result from the selection of the less exacting individuals

which are normally present in the susceptible parent culture (McIlwain, 1943a, b).

Increased resistance resulting from selection often exhibits a remarkable specificity for the toxic substance with reference to which selection has taken place. On the other hand, there are many examples where variation results in the production of variant strains exhibiting resistance to a variety of different agents. Resistance to one type of sulfonamide, for instance, seems to be reflected in resistance to all types of sulfonamides (Davies and Hinselwood, 1943; Kirby and Rantz, 1943). Even more striking is the fact that by growing staphylococci on an agar medium containing a sufficient concentration of penicillin, one can obtain small colony variants of these organisms (G forms) which are resistant not only to penicillin, but also to unrelated substances like methyl violet. Similar small colony variants obtained by growing the parent strain on BaCl_2 agar are also found resistant to penicillin and methyl violet, although they have never been exposed to these inhibitors; in fact, all small colony variants exhibit the same behavior regardless of their origin. In other words, there are certain forms of drug fastness which are entirely nonspecific in their origin and manifestations (Schnitzer, Camagni, and Buck, 1943; Youmans and Delves, 1942).

Although the progressive selection of the few resistant forms normally present in a susceptible culture probably accounts for the occurrence of drug fastness, the nature of the phenomenon is not explained thereby. It is certain, in fact, that resistance can result from many different unrelated mechanisms and cannot be explained in terms of one single theory. At one extreme can be placed the case of those drugs which interfere with growth by competing with some essential metabolite and by causing a condition similar to nutritional deficiency. Resistance to these agents is likely to exhibit a marked degree of specificity when it is the result of an increase in production, or a decrease in requirement, of the metabolite concerned. At another extreme are those alterations of the physicochemical properties of the bacterial cell—expressed in decrease of permeability, for example—which pre-

vent or retard the fixation of inhibitors on the susceptible cellular structures. Although the type of resistance which is thus produced can exhibit marked quantitative differences with reference to the varied types of inhibitors, it is essentially nonspecific in its mechanism. It can be associated with many other modifications of metabolism which thus appear to be correlated with resistance although they do not possess causal relationship to it.

Since so many of the properties and characters of bacteria can undergo independent variation, the modifications which result in drug fastness can occur without any alteration of virulence or can be associated with structural or metabolic variations which affect one or several of the factors of virulence. Too little factual data are available at the present time to permit a complete analysis of the problem. Enough is known, however, to warn against any hasty interpretation drawn from the apparent correlation of drug fastness with other modifications of the cell. Like many other biochemical and physicochemical properties of bacteria, virulence can vary in association with, or independently of, resistance to one or another type of drugs. It is possible, therefore, that the widespread use of chemotherapeutic agents will favor the production of many unsuspected variants, exhibiting all degrees of drug fastness and of pathogenicity.

Alterations of Cellular Structure and Metabolism Correlated with the Development of Drug Fastness in Bacteria.—Many attempts have been made to explain drug resistance in bacteria in terms of the same mechanism invoked to account for the development of resistance in trypanosomes. Indeed, resistant bacteria may differ from the parent susceptible strain by a decreased ability to fix the inhibitor. This is illustrated by relapsing fever spirochetes, the normal forms of which absorb arsenic and gold from neoarsphenamine and solganol respectively, whereas the resistant strains fail to absorb either element (Feldt, 1934; Fischl, Kotrba, and Singer, 1934; Fischl and Singer, 1934). It has also been claimed that increased resistance to the bacteriostatic effect of a basic dye may result from a change in the iso-electric point of the culture under consideration, accompanied by decreased affin-

ity for the dye (Stearn, 1927). In most cases, however, an effort has been made to establish that the phenomenon of drug fastness in bacteria is associated with definite alterations of cellular metabolism.

These biochemical alterations often appear to be of a rather simple nature. Thus, the increased resistance of yeast to formaldehyde or to arsenic can result from an increased production of H_2S or from an increased ability of the resistant cell to oxidize the aldehyde group to a nontoxic product (Effront, 1920). Pneumococci rendered resistant to sulfapyridine dehydrogenate glycerol, lactic acid, and pyruvic acid less rapidly than do the susceptible parent cells, and also produce less hydrogen peroxide during growth; their ability to dehydrogenate glucose apparently remains unchanged (MacLeod, 1939). It would be interesting to know whether the small colony variants (G forms) of staphylococcus mentioned earlier, which are resistant to both penicillin and methyl violet, are also characterized by decreased metabolic activity, a fact suggested by the slower and less abundant growth of these strains (Flynn and Rettger, 1934; Schnitzer, Camagni, and Buck, 1943).

In a number of cases, the metabolic differences associated with increased resistance to certain drugs have been defined in terms of the nutritional requirements of the organisms involved. If sulfonamides owe their antibacterial activity to their ability to compete with p-aminobenzoic acid in cellular metabolism, it should be possible for an organism to become able to grow in the presence of the drug by becoming more efficient in producing or utilizing this essential metabolite. In fact, it has been shown that the sulfonamide-resistant forms of a number of bacterial species produce greater amounts of p-aminobenzoic acid than do the parent strains (Landy, *et al.*, 1943, 1944). Analysis of drug resistance in terms of decreased nutritional requirements is susceptible of a general formulation. Drug fast strains can be regarded as having been trained, *i.e.* selected, so that they possess increased synthetic ability, or decreased needs with respect to the essential metabolites or enzymes with which the drug in question interferes. The pro-

duction of resistant forms by growing the susceptible organism in the presence of increasing concentrations of the drug is essentially the same as a process of nutritional training, in which organisms are grown in the presence of decreasing concentrations of an essential nutrient. Thus, it is possible to render organisms resistant to a drug not only by growing them in its presence, but also by training them to multiply in the absence of the metabolite with which this particular drug exhibits specific competition. Preformed aminocarboxylic acids are normally prerequisites for the growth of *Staph. aureus* and *E. typhosa*, but these organisms can by suitable training be induced to grow in media lacking these amino acids which they are then capable of synthesizing; similarly, aminosulfonic acids are inhibitory to normal cultures of *Staph. aureus* and *E. typhosa*, but do not inhibit the derived strains capable of synthesizing their own aminocarboxylic acids. *E. coli* and *Strept. hemolyticus* inhibited in their growth by acriflavine can be made to grow further by the addition to the medium of nucleotides and of phenylalanine. Increased production of these normal metabolites might therefore render a variant strain of the organism more resistant to the drug. Pantoyltaurine-resistant strains of *Corynebacterium diphtheriae* can be produced without the use of the drug by serial subcultures in the absence of alanine and of pantothenic acid; conversely, strains sensitive to pantoyltaurine can be obtained by repeated subculture in high concentration of pantothenate (Gladstone, 1937, 1939; Knight, 1936; McIlwain, 1941a, b, 1942a, b, 1943a, b, c).

It is obvious, therefore, that many types of variation in metabolic activity can modify the resistance of a bacterial culture to a given substance. On the other hand, it must not be overlooked that resistance can result, not from a change in the metabolic equipment of the cell, but from failure of the inhibitor to reach the susceptible cellular substrate, due to some modification of the plasma membrane or of the cell wall. For instance, the loss of ability to ferment lactose is not always due to a loss of the enzyme lactase, but rather to a change in permeability which prevents the disaccharide from penetrating the cell and therefore

from reaching the enzyme (Deere, 1939). Some of the phenomena of resistance exhibited by trypanosomes can be explained in similar terms, and it is possible that many cases of drug fastness, especially those which do not exhibit great specificity, are due to changes in cellular permeability.

6. CHEMOTHERAPEUTIC AGENTS

... some of the inventions already known are such as before they were discovered it could hardly have entered any man's head to think of; they would have been simply set aside as impossible. For in conjecturing what may be men set before them the example of what has been, and divine of the new with an imagination preoccupied and colored by the old; which way of forming opinions is very fallacious; for streams that are drawn from the springheads of nature do not always run in the old channels. FRANCIS BACON

Selectivity of Chemotherapeutic Agents.—Of the countless antimicrobial substances produced by chemical methods or extracted from biological materials, only a very few can be used in the prevention and therapy of infectious diseases. It would be of the greatest theoretical and practical interest, therefore, to define what property or combination of properties are required to endow a substance with chemotherapeutic activity.

The ideal chemotherapeutic agent is, obviously, one which exhibits great affinity for the parasites but is completely inactive against the constituents of the tissues of the host. On the other hand, most antimicrobial agents, of either synthetic or biologic origin, affect all kinds of living cells, reacting with morphological structures or with metabolic systems common to all living matter, and behave, in other words, as general protoplasmic poisons. It is interesting in this respect that, of the few agents which have proved to retain their activity *in vivo*, several exhibit definite selectivity in their action. Thus, penicillin which is so immensely active against certain types of organisms (Gram-positive cocci and bacilli, gonococci, spirochetes, etc.) possesses little or no activity even *in vitro* against Gram-negative and acid fast bacilli. It does not affect the growth or metabolism of tissue cells, a fact reflected in the remarkable lack of toxicity of the substance

(Chain and Florey, 1944). Gramicidin also exhibits great selectivity as to the microbial species which it affects, and is in general most active against Gram-positive species (gonococci and meningococci being also susceptible). Gramicidin is also very toxic for erythrocytes and for spermatozoa, but is apparently innocuous for the other types of animal cells so far tested (Dubos and Hotchkiss, 1941, 1942); its hemolytic property is sufficient to rule out its use in the treatment of systemic infections, but its lack of toxicity for other cells, especially those involved in tissue repair, may render it useful in the treatment of certain localized infections, as illustrated by the reports of its successful utilization in the treatment of infected ulcers and of bovine mastitis caused by streptococci. Even the sulfonamides are not equally active against all types of cells. Many of the infections caused by Gram-negative bacilli have not responded well to sulfonamide therapy. The organisms of the dysentery group constitute a notable exception to this statement, and it is perhaps of some interest that they are classified by some authors as "Gram-variable" and are more susceptible to basic dyes than many other Gram-negative bacilli.

It is obvious that many factors influence the selectivity of an antimicrobial agent. The acidic and basic properties of the cell under consideration, the nature and property of its membrane, its permeability, the relative importance for metabolism and viability of the specific biochemical systems affected by the antiseptic, the activity of the autolytic enzymes, etc., etc., are all attributes which bear a definite relation to susceptibility. Moreover, each one of these properties can undergo variation either independently or simultaneously and thus give rise to mutant forms of modified susceptibility. It is because so many unrelated factors affect the outcome of the antiseptic and chemotherapeutic reaction that the receptor concept of Paul Ehrlich, although correct in its general principle, has been relatively ineffective for the analysis of the problem. Among the few generalizations which appear justified at the present time, it may be appropriate to mention that, in their behavior towards many types of antiseptics, certain Gram-negative bacilli behave more like animal tissue cells than

do other microorganisms. If confirmed, this view would provide an explanation for the fact that attempts to discover chemotherapeutic agents effective against the salmonella have presented such great difficulties.

Effect of Antiseptics and Chemotherapeutic Agents on Cellular Metabolism.—Most antiseptics interrupt immediately and irreversibly the metabolism of living cells, whether it be measured in terms of oxygen consumption, production of carbon dioxide, acid production, luminescence, etc., and measurement of inhibition of these metabolic events has often been suggested as a quantitative method of determination of antiseptic action. Unfortunately, what little is known of the effect on metabolism of some of the few agents which have been found to retain their activity *in vivo*, suggests that the application of the method is beset with many pitfalls.

Although there is still much argument concerning the intimate mechanism of the action of sulfonamides, it is certain that these drugs do not completely, or even markedly, interrupt catabolism. Bacterial cells inhibited in their growth continue to metabolize even in the presence of an excess of sulfanilamide. Similarly, penicillin does not affect the respiration of staphylococci (Chain and Florey, 1944). The comparative effects of gramicidin and tyrocidine are illuminating with reference to this problem. Both these substances are produced by cultures of *Bacillus brevis*, both are polypeptides in nature, both exhibit antibacterial activity. Tyrocidine is not only toxic for bacteria, but also for all types of living cells so far tested, and is therefore entirely ineffective *in vitro*; gramicidin, on the contrary, although toxic for spermatozoa and erythrocytes, is innocuous for many other types of tissue cells and, among bacteria, affects chiefly the Gram-positive species. Interestingly enough, tyrocidine, like other common antiseptics, completely and irreversibly inhibits cellular metabolism, whereas gramicidin permits maintenance, even though in a modified form, of oxygen consumption, of CO_2 and acid production, and of several other metabolic functions of the most susceptible cells (Chapter VIII:4). It appears, therefore, that the typical

antiseptic behaves as a gross protoplasmic poison, destroying the general metabolic cellular mechanisms. On the contrary, most chemotherapeutic agents have a very selective effect on some specific metabolic steps, although the nature of the specific steps inhibited does undoubtedly vary from one agent to another.

The following quotation from a recent review states the general terms of the problem. "Those properties which seem characteristic of any living cell are (a) the ability to transform various substrates found in a favorable environment into characteristic end-products and to liberate chemical energy in so doing, (b) the ability to use this chemical energy for synthesizing other substrate molecules into the typical constituents of living protoplasm, (c) the maintenance of the organized transforming and synthesizing systems inside a cell membrane or retaining surface, and (d) the ability to transport various needed substrates and metabolic products selectively across the boundaries of the system. As abstract functions these are distinct enough, but as biochemical and biophysical mechanisms, they undoubtedly greatly overlap.

"Most of what we know about bacterial physiology is concerned with property (a) rather than the other, energy-using functions. It is natural, therefore, that each new antibacterial agent is first tested for effect upon these degradative metabolic functions. Typical antiseptics like the mercury, copper, iodine, chlorine derivatives, the phenols and detergents, have been found to interfere with the degradative processes. Evidence has been presented above suggesting that gramicidin affects rather some energy-using process (b) or (d) which would normally allow carbohydrate and phosphate storage. It is beginning to appear likely that effective chemotherapeutic agents like the sulfonamides and penicillin probably also affect the way in which the susceptible cell uses the energy rather than the processes by which energy is liberated" (Hotchkiss, 1944).

Search for New Chemotherapeutic Agents.—The analysis of the differential effect of common antiseptics and chemotherapeutic agents on cellular metabolism is a problem not only of theo-

retical interest, but also of immediate practical importance. Because of the cost and labor involved in animal experimentation, many workers have attempted to develop shortcut "screening" methods for the rapid evaluation *in vitro* of the activity of antimicrobial agents. All of these methods make use of inhibition of metabolism as measured by oxygen uptake, production of carbon dioxide or of acid, luminescence, and other biochemical manifestations in systems consisting of resting or proliferating bacteria. In fact, it has been hoped that these metabolic methods would permit a comparison of the effect of a given drug on the infectious agents and on the tissue cells, and would thus give preliminary information concerning therapeutic indices. The fact that some of the most effective chemotherapeutic agents fail to interfere with respiratory reactions, whereas the common antiseptics completely abolish all metabolism, renders difficult the interpretation of the results obtained by metabolic techniques for the screening of promising substances.

A striking example of the lack of correlation between inhibition of bacterial metabolism and chemotherapeutic value appears in a study of the effect of varied antibacterial agents on the production of light by luminous bacteria (Rake, McKee, and Jones, 1942). It can be seen from the results presented in Table 44 that, of all the substances tested, the ones which are least active in inhibiting the production of light by *Photobacterium fischeri* are the sulfonamides, penicillin, and gramicidin. Interestingly enough, these three substances retain all or part of their antimicrobial activity *in vivo*. In other words, not only is the ability of a substance to inhibit light production by bacteria no measure of its activity as a chemotherapeutic agent, but if any correlation exists at all, it is a negative one.

In vitro methods of evaluation of an antibacterial agent based on the determination of its effect on the overall oxygen uptake or acid production of an organism are adequate in the case of the typical antiseptics which destroy the general metabolic, and especially catabolic, cellular mechanisms. These methods are not applicable, however, to those substances which have a selective

TABLE 41

COMPARISON OF ANTILUMINESCENT AND ANTIBACTERIAL ACTIVITIES AND ALI:AR RATION OF VARIOUS ANTIBIOTIC SUBSTANCES

SMALLEST AMOUNT IN μ G SHOWING ACTIVITY				ALI:AR RATIO	
Antiluminescent Test		Antibacterial Test			
Tolu p-quinone	0.11	Gramicidin	.002	Tolu p-quinone	0.7
Pyocyanase*	3	Tyrothricin	.008	Pyocyanase	0.7
Clavacin* 1	11	Penicillin 2	.0156	Clavacin 1	11
		Penicillin 1	.06		
		Flavatin	.256		
Aspergillie Acid	15	Gramidinic Acid	.21	Sod. Clavacinate	11
Gliotoxin	17	AP21	.31	Clavacin 2	15
Clavacin* 2	22	Actinomycin A	.54	Sulfanilamide	< 30
				Phenol	5
Pyocyanin*	47	Aspergillie Acid	2	Pyocyanin	17
Actinomycin A*	51	Gliotoxin	2.1	Lauryl Sulfate	14
Streptothricin*†	56	Streptothricin	2.8	Aspergillie Acid	15
Sodium Clavacinate*	91	Fumigacin	13	Gliotoxin	2
Flavatin	256				
Fumigacin*	273	Pyocyanin	27	Streptothricin	20
Lauryl Sulfate	273	Pyocyanase	42	Fumigacin	21
Phenol	1170	Tolu p-quinone	55	Actinomycin A	100
				Flavatin	100
				AP21	> 1500
Penicillin‡ 1	1650	Lauryl Sulfate	59	Gramidek	
Sulfanilamide	3940	Clavacin 1	63	Acid	> 2100
				Penicillin 1	2500
Gramicidin‡	> 500	Clavacin 2	113	Tyrothricin	> 6250
Gramidinic Acid	> 500	Sod. Clavacinate	500	Gramicidin	> 25000
Tyrothricin‡	> 500	Phenol	2300	Penicillin 2	> 32000
AP21‡	> 500	Sulfanilamide	> 7000		
Penicillin 2	> 5000				
Fog White	No activity	Fog White	No activity	Fog White	—

* Supplied through the courtesy of Dr. S. A. Wakeman of the N. J. Agricultural Experiment Station, Rutgers University

† Crude preparations

‡ Supplied through the courtesy of Dr. J. C. Hoeghede of the Laboratories of E. R. Squibb and Sons. AP21 is the active material obtained from a growth-inhibiting anaerobe

Data from Rake, McKee, and Jones (1942, Table 1, p. 137)

effect on a specific metabolic step and which prevent growth through some subtle interference with synthetic reactions or cellular division. In fact, it is evident that the metabolic screening

methods would have failed to detect some of the most interesting antibacterial agents. The sulfonamides are much less effective in blocking bacterial oxidoreductions than are many other related aromatic amines and nitroso compounds which are valueless as chemotherapeutic agents; arspenamine acts so slowly against spirochetes *in vitro* that it would not have been considered a promising substance; a great many toxic quinones and phenols produced by fungi, actinomycetes, and bacteria would have been selected in preference to penicillin; of the two antibacterial agents produced by *Bac. brevis*, tyrocidine which immediately abolishes bacterial oxidations would have retained attention rather than gramicidin, which, far from destroying cellular metabolism, stimulates oxygen uptake and acid production of susceptible cells.

If generalizations are justified in the light of the meager information available, it appears that the few substances which retain their antibacterial activity *in vivo* exert upon the susceptible cells a limited, specific injury, probably reversible to some extent, perhaps affecting some synthetic process, or some step in cellular division. The search for new chemotherapeutic agents should not be limited to the mere production, by the methods of organic chemistry or from natural sources, of more and more bacteriostatic and bactericidal substances. It should be guided by an understanding of that property, or more likely combination of properties, which determine whether a certain agent can retain its antibacterial activity *in vivo* without causing irreversible damage to the host. Progress in this direction will depend in part upon an increased knowledge of the steps of bacterial metabolism—catabolic or anabolic—for which there can be developed specific inhibitors exhibiting selective affinity for the parasite.

Although several workers doubt that the sulfonamides owe their bacteriostatic effect to their structural similarity with p-aminobenzoic acid, and to their ability to compete with this metabolite at enzyme surfaces, this hypothesis is still the most widely held and has stimulated the synthesis of other analogues of essential growth factors which also possess antibacterial properties (Chap-

ter VIII:4). Pantoyltaurine, the sulfonic acid analogue of pantothenic acid, is of special interest in the present discussion since this bacteriostatic agent has been shown to retain its activity *in vivo* under certain conditions, and to behave, therefore, as a chemotherapeutic agent. Pantoyltaurine exerts its antibacterial action by competing with pantothenic acid, a substance essential for the growth of many bacteria. Thus the growth of *Strept. hemolyticus*, *in vitro*, is retarded when the ratio of pantoyltaurine to pantothenate concentrations in the medium is above a certain range. Rats infected with virulent streptococci can be protected against many lethal doses (from 10,000 to 1,000,000 L.D.), of these organisms by the repeated injection of doses of pantoyltaurine large enough to maintain in the blood stream a ratio equal to, or greater than, that which is antibacterial *in vitro*. Since, on the other hand, the normal pantothenate level in the blood of mice is much higher than in the blood of rats, it is impossible to maintain in the former animals a pantoyltaurine-pantothenate ratio sufficiently high to inhibit bacterial growth, and the drug cannot exhibit therapeutic activity under these conditions (McIlwain and Hawking, 1943).

Thus, the chemotherapeutic activity of an antibacterial agent is determined, not only by the nature of its action on the parasite, but also by the environmental conditions prevailing in the body of the host. Just as pantoyltaurine is inhibited by the high level of pantothenic acid present in the blood of the mouse, so the sulfonamides are inhibited by the high concentration of *p*-aminobenzoic acid and other inhibitors present in exudates and at the site of tissue breakdown. It is because most of the metabolites essential for the growth of microorganisms are also normal constituents of the body fluids of animals, that many analogues of essential growth factors, although efficient inhibitors of bacterial growth *in vitro*, fail to exert a chemotherapeutic effect *in vivo*.

The practical achievements of chemotherapy have come from the utilization of substances which interfere with the metabolic activities of microorganisms. It should not be forgotten, however,

that it is also possible to alter the pathogenic career of certain bacteria through the enzymic destruction of some of their structural components or products. Enzymes exhibit a high degree of specificity for their chemical substrates. Should, therefore, practical techniques become available for the production in a purified form of enzymes capable of destroying the vulnerable bacterial structures of significance in the infectious process, a new method would have been found to reach selectively the pathogenic agent in the midst of all the constituents and products of the host.

IX

TRENDS AND PERSPECTIVES

"It is a poor sort of memory that only works backwards," the Queen remarked.

LEWIS CARROLL

1. DEVELOPMENT OF KNOWLEDGE CONCERNING BACTERIA

Celsus . . . tells us that the experimental part of medicine was first discovered, and that afterwards men philosophized about it, and hunted for and assigned causes; and not by an inverse process that philosophy and the knowledge of causes led to the discovery and development of the experimental part. And therefore it was not strange that among the Egyptians, who rewarded inventors with divine honors and sacred rites, there were more images of brutes than of men; inasmuch as brutes by their natural instinct have produced many discoveries, whereas men by discussion and the conclusions of reason have given birth to few or none.

FRANCIS BACON

WE have considered in succession the nature and properties of several structural components and products of the bacterial cell, their reactions with the environment, and the significance of this knowledge for the understanding of some of the phenomena of the infectious process. This arrangement of the subject material has the merit of convenience and the appearance of a certain formal logic. It does not express, however, the logic of life, the historical sequence of the discoveries which led to our present points of view.

It is true that bacteria were first seen and described in morphological terms by biologists who were interested in them as new forms of life, without much regard to their activities. Nevertheless, it is unlikely that much would be known today of the nature and behavior of bacteria, had interest in their morphology remained the only incentive to their study. The great productive period in bacteriology did not begin immediately with the recog-

nition of microorganisms as living objects, but only when it was realized that bacteriological phenomena constitute events of great importance to man. It was the appreciation of the immense role played by bacteria in the economy of nature, of the intensity and variety of their activities—resulting either in the transformation of organic matter or in the phenomenon of parasitism—which captivated the attention of the chemist, biologist, and the physician as well as the layman. This emphasis on bacteriological “events” rather than on the bacteria themselves determined to a marked extent the problems and the methodology of the new science. It probably influenced also the selection of a certain type of investigator attracted by the dynamic rather than by the intellectual aspects of the new problems, and perhaps more eager to do something about them than concerned with the understanding of their nature. In any case, the first period of the bacteriological era is characterized by the discovery of a number of phenomena and techniques which are related to immediate practical problems, and which often do not exhibit any obvious relation to the contemporary biological sciences. By the beginning of the twentieth century, bacteriology was following an independent course, almost uninfluenced by the doctrines and methods of classical biology.

It would be interesting to identify the forces and the individuals that were influential in modifying the trend of bacteriological philosophy and methodology during the present century. Many investigators probably believed that the most immediate practical problems had been solved, or at least that the somewhat primitive pioneering methods had given all the practical results that could be expected of them. It was also realized that many of the official viewpoints and practices had been accepted without critical evaluation of their validity and practical value, and that the time had come to rebuild the structure of the science on more rational and secure foundations. Whatever the influences at work, there has been an obvious tendency during the past few decades to restate the bacteriological problems in terms of the classical sciences and of the prevalent biological, physiological, and bio-

chemical philosophies. It is hardly necessary to call attention to the ever-increasing number of publications in which bacteria are studied, and bacteriological phenomena analyzed, by the standard physiological and chemical methods. These studies belong so clearly to the tradition of experimental biology and biochemistry that they naturally find their place in the standard journals of these sciences.

In addition to the general phenomena of cellular biology, however, bacteriology has emphasized other phenomena which are determined by the particular properties of the bacterial cells, and especially by their behavior as parasitic agents. It is also characteristic of bacteriology that much of the theoretical knowledge peculiar to it was the by-product of the solution of practical problems by empirical methods. In many cases, successful practices were developed before there was any understanding of their rational basis. Indeed, it was the analysis of the mode of action of these empirical methods which led to the recognition of a number of reactions between bacteria and the environment, and to the subsequent description of the components of the cell involved in these reactions. Since much of the knowledge of cellular organization which it has been the purpose of this book to discuss is the result of this process of "discovery in reverse," it may be justifiable to illustrate it again by a few examples.

When antityphoid vaccination became an established practice early in the present century, practical considerations led to the use in the preparation of the vaccine of cultures of the typhoid bacillus which formed stable bacterial suspensions in saline. It is now known that these stable suspensions are given chiefly, if not solely, by cultures in the smooth specific dissociative phase. Analysis of the mechanism of immunity eventually revealed the importance of the O antigen characteristic of this phase, and against which is directed the protective antibody. Only later was it recognized that the specific part of the O antigen is a polysaccharide which exists in the cell as a molecular complex probably present at or near the surface, and which, when isolated in solution, possesses many of the toxic and immunogenic characteristics of the

whole bacterium. Similarly, analysis of the therapeutic effect of antipneumococcal serum led to the discovery that the effective antibody reacts with the capsular material present in virulent pneumococci. The *in vitro* reaction between antibody and capsular substance served as a guide for the isolation of the polysaccharide constituent of the latter, and eventually revealed the role of specific carbohydrates in immune reactions. Equally characteristic of this progression from practical achievement to theoretical concept is the growth of knowledge which resulted from the discovery that an azo dye, prontosil, exerts a chemotherapeutic effect on bacterial infections. The dye itself was soon found to be inactive against bacteria. Fortunately, prontosil is reduced in the animal body to sulfanilamide, the substance which is now known to be responsible for the chemotherapeutic activity of the dye. Subsequent studies revealed that the antibacterial effect of sulfanilamide is inhibited by p-amino-benzoic acid. The two substances being related from the point of view of molecular structure, it was deduced, and eventually established, that p-amino-benzoic acid is an essential metabolite with which the drug apparently competes.

Thus, practical problems were often solved by empirical methods without any knowledge of the theoretical factors involved. Since much of bacteriological knowledge was derived from the subsequent analysis of the mode of action of these empirical methods, one may wonder whether theoretical analysis has any justification beyond the intellectual esoteric satisfaction of the investigator. It is hardly necessary to emphasize that the more complete the understanding of the mode of action of a practical procedure, the better can we control it and the more effective becomes its performance. It will suffice to mention in this respect the great improvements in the methods of preparation of the vaccines used for active immunization or for the production of therapeutic sera, which resulted from increased knowledge of the cellular factors concerned in the immunity process.

It is also obvious that theoretical understanding of a problem often facilitates, by a process of analogy, the application of a

discovery to other related situations and opens new vistas which in turn lead to new problems. Thus, the discovery that diphtheria toxin can be transformed into a toxoid, which is no longer toxic while retaining its power to elicit the production of neutralizing antibodies, is immediately applicable to the production of toxoids from a few other toxins by the use of identical methods. This finding also demonstrates that it is possible to alter the physiological activity of a substance without destroying its specific antigenicity, a general point of view which can be transferred to many other biological problems.

Another pattern of progress consists in the prediction of new phenomena by logical deduction from a thorough knowledge of the properties and behavior of the material under investigation. Only sciences in a reasonably advanced state of development offer opportunities for such rational predictions. To bring bacteriology to this state, it is necessary to abandon the anthropomorphic attitude which characterized earlier efforts; bacteria must be studied, not only in the effects which they have on practical human problems, but also for what they are and what they do as independent living organisms. The classical methods of cytology, physiology, and chemistry are obviously contributing fundamental information of this nature, but in addition to this knowledge there are phenomena, methods, and concepts, which are peculiar to bacteriology and which have evolved chiefly from two striking properties of bacteria; the specificity of the reactions which they induce and the great variability of the bacterial cell. It appears appropriate, therefore, to conclude our survey by a general review of the nature and significance of these two properties.

2. NATURE OF SPECIFICITY

All flesh is not the same flesh: but there is one flesh of men, and another flesh of beasts, and another flesh of birds, and another of fishes.

ST. PAUL, I CORINTHIANS, XV, 39

In 1857, on the occasion of his first paper dealing with the microbial theory of fermentation, the "Mémoire sur la fermentation appelée lactique," Pasteur expressed his belief that, for each

type of fermentation, one would find a specific ferment, characterized by its morphology and physiology as well as by its specific behavior as a chemical agent. It was the same year that Pasteur reported for the first time on a microbial agent capable of attacking the d-form of tartaric acid, but practically inactive against the l-form. Thus, the great specificity of the biochemical reactions induced by microorganisms was recognized at the very beginning of experimental microbiology, and it is well known that subsequent advances have provided many other illustrations of this property. There are for example bacteria which oxidize ammonia to nitrites and others which oxidize nitrites to nitrates; the ability to decompose certain complex polysaccharides such as cellulose, or the capsular substances of pneumococci, is the fairly exclusive property of a few bacterial species. As a first approximation, therefore, it appears justified to state that, under natural conditions, each one of the microbial species concerned in the economy of organic matter, is more or less specifically adapted to the performance of a limited, defined biochemical task.

Parallel with the doctrine of biochemical specificity of microorganisms, and even more spectacular in its claims, grew the dogma of the specific etiology of infectious diseases. The isolation by Pasteur, Koch, and their disciples of the causative agents of many infectious diseases soon led to the view that each kind of infection is caused by one and only one kind of microorganism. Etiology, rather than pathology and symptomatology, was assumed by many to be the only rational basis for the classification of disease.

Finally, specificity found an even more convincing expression in the phenomena of immunity, whether observed *in vivo* or analyzed in terms of *in vitro* reactions. Animals recovered from infection with one microbial agent exhibit specific resistance against infection with the same agent. Furthermore, the immune animal produces antibodies capable of differentiating not only between the different bacterial species, but even between strains of the same species. These antigen-antibody reactions have been made the basis of exquisitely sensitive diagnostic methods and

have led to the development of specific prophylactic and therapeutic procedures. In Ehrlich's terminology, specificity is due to the presence in the bacterial cell of a multiplicity of receptors, each one endowed with specific affinity for different dyes, antiseptics or antibodies.

Thus, the concept of specificity has been, from the beginning, one of the main driving forces in the development of bacterial biochemistry and of the theories of infection and immunity. Although time has not altered the general terms of this concept, further analysis has greatly sharpened our understanding of its significance. Specificity is no longer referred to the bacterial cell as a whole; it is the summation of the multiple specificities of the many components and attributes of the cell. Surprisingly enough, Pasteur, trained as a chemist, came to regard the faculty to cause alcoholic fermentation as a property of the "living" cell, in fact, as synonymous with life. This belief did not arise from his failure to conceive that "soluble ferments" can cause alcoholic fermentation. He had tried to isolate these soluble ferments and had failed as others subsequently failed with the strain of Paris yeast which he used. Although Claude Bernard expressed on several occasions his belief in the chemical enzymic theory of fermentation, it was Büchner who first obtained from Munich yeast a soluble "zymase" preparation which produced alcohol from sugar in the absence of living cells. This discovery symbolizes the shift in the concept of biochemical specificity from the cell as a whole, to its active components. Biochemical activity is now described in terms of the individual cellular enzymes and of the integration of the reactions which they induce into an orderly and controlled physiological system. The further evolution of our concept of enzymic action has resulted in an even sharper definition of specificity. Enzymes enter into a definite combination with the substrates which they attack, and the specificity of the enzymic reaction is conditioned by the molecular configuration of the substrate which, because of its structural compatibility with the homologous radical in the enzyme molecule, permits a union based on the familiar "lock and key" relationship. Biochemical speci-

ficity, first considered as a property of the whole cell, then of a constituent of the cell, has now been traced to a determinant molecular radical of that constituent.

The concept of immunological specificity has undergone an evolution parallel to that of biochemical and enzymic specificity. It was first recognized that bacterial species can be differentiated by the fact that they do or do not agglutinate in the presence of proper dilutions of immune antisera. These same antisera were then found to precipitate soluble extracts of the bacterial cells which they agglutinate. Finally, the demonstration that each cell contains several antigenic constituents revealed that the immunological behavior of an organism is the summation of the immunological activities of its individual components. As in the case of the enzyme-substrate reaction, the union between antigen and antibody is conditioned by the presence in the antigen molecule of determinant groups capable of determining both the occurrence and the specificity of the reaction. Thus, the concept of immunological specificity has progressively shifted from the bacterium as a whole, to the different molecular constituents of the cell, and finally to the determinant radicals of these molecules.

Immunological specificity exists at different levels, so to speak, in any bacterial culture. There are specific substances which vary from strain to strain within one group; such are the M proteins of group A hemolytic streptococci or the capsular polysaccharides of pneumococci; similarly differential races of bacteriophage separate the typhoid bacilli into several specific substrains, etc. On the other hand, each larger group of bacteria contains certain antigens which are characteristic of the group and which define it immunologically. The existence of these group specific substances is of obvious importance for bacterial taxonomy, and we have seen that these substances may serve eventually to define evolutionary lines on the basis of serological relationships. It may be worth reiterating also that some of the group antigens may be of significance in the problem of immunization.

The favorable results obtained by the specific serum therapy of pneumococcus pneumonia has focussed the attention of in-

investigators on antibodies directed against the type specific substances, and subsequent advances have justified this emphasis. For type specific therapeutic sera to be used successfully, however, it is necessary that the specific type of the organism responsible for the disease be established by adequate tests, in order to permit the selection of the proper serum. The preventive immunization of whole populations presents an entirely different problem. Under practical field conditions, pneumococci, streptococci, dysentery bacilli, etc., exist in a large number of types, many if not most of which are capable of causing disease; there is, furthermore, no way of predicting the relative prevalence of the different types in any outbreak. Since it is practically impossible to establish an effective level of immunity against all the different pathogenic types, there is reason to believe that immunization of threatened populations with type specific vaccines is an impossible goal. It is not unlikely that the practical success of antityphoid vaccination may be due in part to the fact that, in spite of minor differences, the strains of typhoid bacilli isolated from different outbreaks all possess essentially the same immunological constitution.

There exists, fortunately, a kind of immunity which transcends the limit of type specificity and which is effective against all the strains of one given bacterial species. Thus, it is possible to immunize experimental animals against pneumococcus infections under such conditions that the immune response is directed against a component of the bacterial cell which is common to all pneumococcus types. Similarly, one could probably find in all bacterial groups analogous cellular components, different from the type specific antigens and capable of giving rise to non-type specific protective immunity. Indeed, the effective immunity which follows the injection of attenuated non-virulent filterable viruses or bacteria may well be due to the non-specific protective antigens which we are discussing, and which persist in the microorganism even after the factors essential to virulence have been lost. It must be acknowledged that the level of immunity achieved by non-specific immunization is usually lower than that which results from the injection of type specific vaccines. Since, on

the other hand, the attention of investigators has been focussed almost exclusively on the latter substances, it remains possible that intensive investigation directed in the proper channels would lead to the development of improved methods of non-specific group immunization.

Antibodies are not the only agents which can react *in vivo* with well-defined cellular structures; certain enzymes, or bacteriophage lines, have, for instance, proved effective in various experimental infections. Whether or not they eventually prove useful as therapeutic agents depends upon factors which cannot be discussed here. In any event, whatever the nature of the therapeutic agent under consideration, it is important to remember that the type specific components of the cell are not the only ones against which can be directed an attack resulting in therapeutic effect. From the practical, as well as from the theoretical viewpoint, the cellular constituents which characterize the larger bacterial groups deserve more attention than they have received heretofore.

There is another aspect of specificity which has received only scant attention from bacteriologists. As is well known, bacterial toxins and poisons exhibit an immunological specificity conditioned by the determinant group of the molecule which stimulates the antigenic response. This specificity can be demonstrated by flocculation and precipitation tests as well as by active and passive immunity. Bacterial toxins, on the other hand, exhibit another kind of specificity which determines the type of cells or the metabolic functions which they affect. Thus, the toxin of the Welch bacillus probably owes its lethal properties to the fact that it hydrolyzes the phospholipid, lecithin, an essential constituent of many animal cells. In general, however, nothing is known of the primary physiological and biochemical lesions caused by bacterial toxins; whether they act by destroying tissues or cellular structures, or by inhibiting essential metabolic functions. Knowledge of the initial reaction which takes place between the toxin and its susceptible substrate would permit a more accurate definition of the specific physiological functions which are disturbed during the infectious process. It would undoubtedly con-

tribute to a better understanding of the pathological and clinical manifestations of the diseases, and would in the long run suggest specific therapeutic measures.

Finally, as we have seen, biochemical specificity forms the rational basis of chemotherapy. Effective chemotherapeutic agents do not behave as gross protoplasmic poisons which affect indiscriminately the structure and activities of all living cells. They interfere selectively with some specific steps concerned in the nutrition, synthesis or cell division of the parasite; other therapeutic agents may react selectively with well defined structural components essential to the pathogenic behavior of the cell.

Specificity, then, is not limited to a few immunological reactions. It concerns all those structural, biochemical, and physiological characteristics which differentiate one organism from another. Knowledge of these distinctive characteristics permits the development of effective therapeutic measures which aim selectively at the parasite or its products amidst the tissues of the host.

3. SIGNIFICANCE OF BACTERIAL VARIABILITY

Admitting that vital phenomena rest upon physico-chemical activities, which is the truth, the essence of the problem is not thereby cleared up; for it is no chance encounter of physico-chemical phenomena which constructs each being according to a pre-existing plan, and produces the admirable subordination and the harmonious concert of organic activity.

There is an arrangement in the living being, a kind of regulated activity, which must never be neglected, because it is in truth the most striking characteristic of living beings . . .

CLAUDE BERNARD

The doctrine of bacteriological specificity found its most general expression in the statement that each species of bacteria is unchangeable in form and in properties, and cannot transform into another species. There is no doubt that the dogma of the fixity of bacterial species was a very necessary discipline at the beginning of the science, and helped to emphasize that many of the claims of the school of pleomorphism were based only on faulty experimental techniques. It is also true, however, that too narrow an

interpretation of this dogma led to the neglect for almost fifty years of one of the most intriguing and most important characteristic properties of the bacterial cell, namely its plasticity, its ability to undergo reversible modifications under the influence of the environment, as well as hereditary transformations independent of the environment. Although evidence of variability had been recognized very early, it was not until the second half of the nineteenth century that the phenomenon received widespread recognition. Awareness of the plasticity of the bacterial cell suggested powerful and original techniques for the study of cellular organization and provided new points of view which define to a large extent the place of bacteriology among other biological sciences.

There is no conflict between the doctrine of variability and the dogma of fixity of bacterial species. Although bacteria vary so readily and so widely under the influence of the environment, or as a result of mutations and other discontinuous modifications, the range of their variation is not unlimited. With enough patience and skill, one could obtain experimentally the different genotypic and phenotypic expressions of a given culture, and thus describe it in terms of its potentialities. In general, therefore, bacteria do not differ from other cells which also can vary by mutation and also respond to a modification of the environment by modifying their behavior. There are a number of factors, however, which differentiate bacterial variation quantitatively, if not qualitatively, from similar phenomena in other biological material.

Bacteria multiply extremely rapidly, giving rise in a short time to very large numbers of generations, a property which gives to selection the chance of rendering evident any form differing appreciably from the norm of the parent culture. Furthermore, growth in broth, and especially in a colony on solid media, corresponds to an enormously high density of cell population. The selective exhaustion of certain nutrients and the accumulation of metabolites and breakdown products, brings about a progressive and profound alteration of the composition of the medium and of the gaseous environment. The continuously changing spectrum of circumstances thus provides ideal conditions for the mani-

festation of the multiple potentialities of the cell. Finally, and probably most important, there is the likelihood that the organization of the bacterial cell is not as rigidly defined as that of higher organisms. Whereas, in the latter, most mutations appear to be lethal, and any marked change in the environment inhibits growth or cell division, it seems that, in bacteria, a wider range of variation remains compatible with the orderly and successful integration of the countless phenomena of growth and metabolism essential to maintenance of life.

Since the different components and properties of the bacterial cell can vary independently of each other, it is possible to obtain a large number of variant forms which, as we have repeatedly emphasized, can be used as reagents in the analysis of bacteriological phenomena. Virulence, in particular, can thus be shown to be the summation of a multiplicity of independent attributes, and no parasite can establish an epidemic state unless it possesses all of these attributes at the same time. Knowledge of the factors of virulence contributes to a better understanding of the cause, nature and course of the disease, and consequently to its more intelligent management. Moreover, by following the changes which occur in a population, not only in the number of pathogenic agents, but also in those properties which determine the virulence of the pathogens, it may become possible to obtain a complete description of the epidemic pattern of infections. When more is known of the nature and variability of the factors of virulence, it will become possible to forecast the course of epidemics, instead of only describing them in historical and statistical terms.

The diversity of forms and properties under which a given bacterium can manifest itself—as a result of reversible effects of the environment or as an expression of hereditary modification—greatly increases the difficulties attending any system of classification. Because the criteria used for the description of plants and animals are not applicable, it is necessary to use descriptive characters such as biochemical activity, presence or absence of flagella or capsules, antigenic constitution, etc., which are precisely those which have been found to undergo variation. Descrip-

tion of a bacterial species limited to that form and those properties which happen to be most stable under the condition of laboratory cultivation obviously is not a sufficient basis for adequate classification and for the discovery of the interrelationship between species and strains. Unless it is eventually proved that bacteria possess a sexual stage, and unless adequate descriptions of the reproductive apparatus become available, the progress of bacterial taxonomy and the study of evolutionary trends among these organisms will require that cultures be described in terms of their multiple potentialities and not of an accidental phenotype.

Much has already been learned of the comparative structure and properties of the different forms in which one given bacterium can exist. We have shown indeed that the combined utilization of cytological, immunochemical, and enzymic reactions has given rise to a methodology which places bacterial cytology in a unique and favored situation. The comparative study of the different variants of one given culture has so far been largely limited to their morphological structures, but there is little doubt that it could apply also to their biochemical processes. It would be interesting to know, for instance, whether the absence of the type specific polysaccharides or proteins in certain variants of the salmonella, pneumococci, streptococci, etc., is due to the fact that these substances are not produced, or to the fact that they are metabolized further and thus cannot accumulate. Although it is usually stated that the nonspecific variants have *lost* the ability to produce the O antigens, or the capsular polysaccharides, or the M proteins, it has not been ruled out that, on the contrary, the loss variation is on the part of the specific variants which can no longer metabolize these substances. The small colony variants (G forms) produced by many bacterial species present also an intriguing problem of biochemical variation. These "G forms" develop very slowly, give on agar or in broth sparse growth consisting of very small cells, instead of the rapid and luxuriant multiplication exhibited by the usual forms of the same cultures. Understanding of the nature of the G variation requires a definition of the metabolic deficiencies which account for this subnormal

growth. Of even greater interest is the fact that an organism can successfully maintain the integration of the metabolic events essential for life after undergoing such profound biochemical modifications.

This is indeed the most striking phenomenon revealed by the study of bacterial variability. The cell can successfully continue to exist and multiply as an independent living object after having lost a great variety of structures and functions which had appeared to constitute important components and attributes of the "normal" parent form. These structures and functions can be lost and regained independently of each other, without altering the essential nature of the germ, or the potentialities of the cell. It is even possible to substitute experimentally one character for another, to cause, for instance, a strain of pneumococcus to produce, and to transfer to its progeny the ability to produce, a polysaccharide different from the one it had been known to synthesize heretofore. Not only does the cell appear as an integrated complex of independent characters, but it is possible to substitute for one of these characters another one homologous, but different, without interfering essentially with cellular organization.

All living objects, whatever their nature or dimensions, obey the same natural laws; it is not to be doubted that the study of bacteria, like that of other cells, will progress with the understanding of the physico-chemical phenomena which are the manifestations of their living processes. But each science has, in addition to that fund common to all departments of knowledge, its particular genius determined by the peculiarities of the material which it studies. The extraordinary plasticity of bacteria, the ease with which they adapt themselves to the environment, either by reversible modification, or by hereditary variation, has not only determined their importance in the economy of nature; it also makes them ideal objects for the study of that organization and integration of independent characters which define and characterize life.

ADDENDUM

NUCLEAR APPARATUS AND CELL STRUCTURE OF ROD-SHAPED BACTERIA

BY

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1. Nuclear structures in rod-shaped bacteria from young cultures
2. Structure of resting bacterial spores
3. Nuclear changes in germinating spores
4. *Cell division in the genus Bacillus*



ADDENDUM

NUCLEAR APPARATUS AND CELL STRUCTURE OF ROD-SHAPED BACTERIA

By C. F. ROBINOW *

1. NUCLEAR STRUCTURES IN ROD-SHAPED BACTERIA FROM YOUNG CULTURES

The search for the nucleus of the bacterial cell has been a lengthy one; its progress has at intervals been critically reviewed in detail, notably by Dobell (1911), A. Meyer (1912), Gotschlich (1927), Pietschmann (1931), Knaysi (1938), and Lewis (1941, 1942), in whose articles ample references to the literature will be found.

Such truth as there was in some of the older observations has, on the whole, failed noticeably to influence the widely held idea of the undifferentiated nature of the bacterial cell. It is only in the course of the last ten years or so that the more or less uniform results of several independent observers have provided convincing evidence of a discrete nuclear apparatus in the cells of many well known species of bacteria.

The work of Badian (1930, 1933, 1935) on *Myxococcus virescens*, *B. subtilis*, *B. mycoides*, and *B. megatherium* made it probable, and Piekarski (1937, 1939, 1940) as well as F. Neumann (1935, 1941) working with *Salmonella schottmuelleri*, *Escherichia coli*, *Proteus vulgaris*, *B. mycoides* and other well known species,

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established the fact that growing bacteria from young cultures contain regular numbers of discrete chromatinic structures going through an orderly cycle of growth and division correlated with the growth cycle of the bacteria.

Badian described the chromatinic bodies as short, more or less dumbbell-shaped rodlets, multiplying by longitudinal division *at right angles* to the long axis of the bacterium and regarded them as chromosomes.

Piekarski's and Neumann's idea of the chromatinic elements was that of homogeneous, round bodies ("nucleoids") which divide by elongation *in the direction* of the long axis of the bacterium, followed by constriction hour-glass fashion.

My own observations (1942, 1944) confirmed those of both Badian and Piekarski and I pointed out that the words "chromosomes" and "nucleoids" refer to two different aspects of the same thing, the latter being optically unresolved groups of the former.

Various factors have long delayed recognition of the morphological organization of bacterial cells. Two of these were particularly powerful: the lack of visible structure in living bacteria from young cultures and the strong affinity of their cytoplasm for basic dyes after fixation.

Formerly, the uniform staining of bacteria was naturally taken as reflecting the homogeneous appearance of the living organism; now we know that the nuclear structures in growing bacteria from young cultures, although they are stained by basic dyes, are obscured by the relatively large amount of stain taken up by the basophilic cytoplasm.

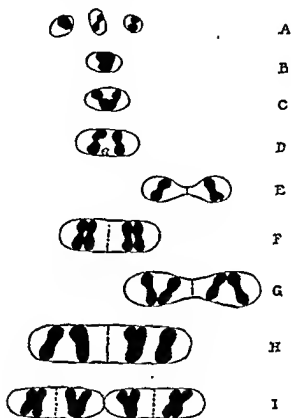
The difficulty inherent in the staining properties of the cytoplasm has been overcome in different ways by different workers. Badian stained bacteria with Giemsa's solution and differentiated the chromatinic structures from the cytoplasm with a 1% watery solution by eosin. Good, if somewhat uneven, results are obtained by this method, but it will not work with spores. Piekarski relied chiefly on the selective action of the Feulgen process. I have used a third method, originally introduced but never sys-

tematically employed by Piekarski (1937). This author mentions that, after treatment with N/1 HCl at 60° C., nucleoids can be demonstrated in the cytoplasm of *Salmonella schottmueleri* with Giemsa's stain, although the cells of this organism are uniformly stained by Giemsa solution without this preliminary treatment in acid.

In my experience, the HCl-Giemsa method has proved more fruitful than the Feulgen process, especially in the study of resting and germinating bacterial spores. In pre-treated Giemsa preparations the chromatinic structures are more deeply stained and the outlines of the bacteria as well as their internal cell boundaries are more distinct than in Feulgen preparations; in addition to these advantages, the staining of hydrolysed preparations with Giemsa solution takes about one tenth of the time required for the Feulgen process. The chromatinic structures appear larger in Giemsa than in Feulgen preparations, but constant comparison has shown a close correspondence between the structures stained by the two methods. After treatment with HCl, Loeffler's methylene blue may be used instead of Giemsa solution (I. M. Lewis 1942); with this stain differentiation of chromatinic bodies and cytoplasm is as clear and sharp as in Giemsa preparations and the lesser brilliancy of the methylene blue is better for photography.

The photographs described in the first part of this chapter are from preparations that were fixed in osmium tetroxide vapour (sometimes followed by sublimate alcohol), treated for 7-10 minutes with N/1 HCl at 60° C. and stained with Giemsa solution. Most of the preparations were photographed mounted in water, but those of *B. mycoides* are from balsam mounts. In making the pictures I have used an Ediswan pointolite lamp in conjunction with a Watson-Conrady condenser, a Beck achromatic substage condenser, n.a. 1.3 (oil immersed), a Zeiss apochromatic objective 90, n.a. 1.30, and a Zeiss compensating eyepiece 15x. Taken at an initial magnification of 2300 times, the pictures were later enlarged to not more than twice their original size. Most of the photographs suffer from an exaggeration of the con-

trasts present in the stained preparations and also from a broadening, through halation, of the chromatinic structures which makes them appear plumper than they really are relative to the size of the cells.



Escherichia coli

A. Single cells, as found in 18 hours slant cultures and during the first hour after transfer to fresh agar plates.

B \rightarrow I. Diagrams illustrating typical aspects of resting and dividing chromosomes in growing bacteria from young cultures. D \rightarrow E, D \rightarrow F and F \rightarrow G, F \rightarrow H are alternative modes of development, F \rightarrow H \rightarrow I being that most commonly followed in young cultures. Cf. Plate 1, figs. 1-7; Plate 2, fig. 13.

The nuclear structures of bacteria are best studied in preparations made during the first few hours of growth after the transfer of cells from old cultures to a fresh nutrient medium.

Beginning with *Escherichia coli* and *Proteus vulgaris*, we find

the cells of old cultures (*i.e.* of 18 hr. incubation and older) are too small to permit of optical resolution of their chromatinic structures. During the increase in the size of the cells that takes place during the lag phase of multiplication, the chromatinic structures become more readily distinguishable from the cytoplasm and are soon found in various stages of division. The basic chromatinic element is a more or less dumbbell-shaped rodlet which divides lengthwise in a plane more or less parallel with the short axes of the bacterium, one dumbbell giving rise to two whole daughter dumbbells (cf. text fig. 1).

Cocoid cells with a single, more or less dumbbell-shaped, chromatinic body are common in very young subcultures of *Proteus vulgaris* (cf. Plate 1, fig. 8), but are rare in *Escherichia*. The first chromatinic structures to become clearly differentiated from the cytoplasm in cells of *Escherichia coli*, awakening on a fresh nutrient medium, are usually compact and apparently already double (Plate 1, fig. 1). The arrangement and the numbers of resting and dividing chromatinic elements in fully developed growing colon bacilli are, however, at present most satisfactorily explained on the assumption that here too a single resting cell possesses but a single chromatinic body.

A faintly stained round granule is often seen between chromatinic bodies at "telophase" (Plate 1, fig. 1f, fig. 2, fig. 8). It is possible that this represents the beginning of the plasma membrane that is formed across the cytoplasm after the division of the chromatinic bodies, but this is not certain. The first division of the chromatinic rodlet is sometimes immediately followed by constriction of the bacterium, especially in *Proteus*, but usually division of the bacterium is deferred until after one or two further divisions of the chromatinic structures. In other words: dividing bacilli may possess 2, 4, or 8 chromatinic bodies and there is no rigid correlation between the onset of constriction of a bacterium and a particular stage in the division of the chromatinic structures. This fact will become more intelligible after a discussion of cell division in the second part of this chapter.

For the reader who has followed the division processes in the photographs of *Escherichia* and *Proteus* on plates and in the diagram text fig. 1, the development of the first few generations of vegetative cells in *B. mycoides* Fluegge and its variant "1110" (Plate 2, figs. 14, 15 and Plate 3, fig. 16) will require no further explanation. Division stages of the chromatinic bodies are usually more easily resolved in *Escherichia* and *Proteus* than in *B. mycoides*, but of the basic similarity of their chromatinic elements there can be no doubt.

Predominance of bacilli with two or four symmetrically spaced groups of chromatinic bodies has been found to be characteristic of the early growth on agar plates of all species of rod-shaped bacteria so far investigated (cf. Plate 2, fig. 13). When cultures get older, specific deviations from the standard pattern gradually appear. Examples are: the long winding filaments with up to thirty regularly spaced groups of chromatinic bodies in swarming colonies of *Proteus vulgaris*, the enigmatic fusion of the chromatinic contents of many separate bacilli joined together at their tips in the centre of "star clusters" of *Phytomonas tumefaciens* (Stapp 1942), the single central chromatinic structure in the rod-shaped cells characterizing 2-3 weeks old cultures of *Phytomonas* spp. (cf. Stoughton 1929 and Plate 5, fig. 34, this paper), finally, contrasting with the last example there are the "longish filaments which show only a few transverse septa and contain dispersed in each intersepted part many nuclear structures of dumbbell shape" which E. Klieneberger-Nobel (1944) regularly encountered in 5-12 hours old cultures of *Clostridium oedematiens* and other anaerobes (Plate 5, fig. 33). It should be added that in even the youngest cultures of aerobic spore-formers and of non-sporing organisms, certain peculiar types of cells are regularly encountered in small numbers, which do not fit into the scheme of development outlined in text fig. 1. The chief characteristic of these elements, which may reach several times the length of normal bacteria, is a dense concentration of chromatinic bodies in the centre of the cell. These forms have been described elsewhere (Robinow 1944) and, since little is

2. STRUCTURE OF RESTING BACTERIAL SPORES

Ever since they were first recognised as the resting stages of bacterial cells some 70 years ago (Cohn 1876), bacterial spores have commonly been regarded as morphologically homogeneous concentrations of protoplasm, and it is only during the last fifteen years that many different observers have described discrete, Feulgen-positive, chromatinic structures in resting spores, usually in the periphery of the cell (Pietschmann and Rippel, 1932; Milovidov 1935, Stille 1937, Schaede 1939, Piekarski 1940, Stapp 1942).

In a study of the behaviour of the chromatinic structure during spore germination (Robinow 1942), I have previously described it as a single, dumbbell-shaped chromosome closely attached to the outer surface of the spore's main body of cytoplasm and somehow fitted into the narrow space between the cytoplasm and the inner surface of the outer surrounding spore membrane.

In further work, I have since confirmed my previous observation that the chromatinic body of the resting spore gives rise to the chromosomes of the vegetative cell, but its dumbbell-shape I have now recognised as the lateral aspect of a flat circular body corresponding to a nucleus rather than to a chromosome.

For my observations I have mainly used spores of two species, *mycoides* 1110 and *B. mesentericus*; repeated comparison of spores of *B. mycoides* Fluegge, *B. cereus*, *B. megatherium*, *B. anthracis* has revealed essentially the same organisation. *mycoides* 1110 is one of the bacteriophage-producing strains of aerobic spore-forming bacteria from soil described by den den de Jong (1933, 1934) who classified it as a strain of *mycoides* because of the selective lytic action of its phage on all typical *Mycooides* strains. The usefulness of this bacillus cytological investigation lies in the size of its spores which are smaller, on the average, than those of *B. mycoides* Fluegge and *B. cereus* although unlike them in every other respect.

The nuclear transformations accompanying spore formation are complex; they are the subject of a study by Dr. E. Klieneberger-

by Knaysi in a new species of a yellow coccus. The latter author has made detailed observations on the mode of division of these granules and found that they divide by simple constriction, hour-glass fashion. In collaboration with Mudd, Knaysi (1943), using an electron microscope, has also observed alleged nuclear bodies in gonococci and staphylococci. No nuclei were seen in cells from very young cultures and the authors concluded that young cells actually lack discrete nuclear structures. This has not been my experience with cells from young cultures of rod-shaped bacteria, and in this connection it should also be remembered that Piekarski and Ruska (1939) were equally unsuccessful in their attempts to demonstrate with the electron microscope nuclear structures in young vegetative forms of aerobic spore-formers where the Feulgen, OsO_4 -HCl-Giemsa and the Bouin-Giemsa methods have, in fact, shown nuclear structures to be present and to be more clearly differentiated from the cytoplasm than in cells from the later stages of the vegetative growth cycle.

It is a remarkable fact, emphasised also by F. Neumann (1941), that vegetative bacteria do not seem to be able to form resting nuclei. In growing bacteria recently divided chromosomes proceed to divide again without first going through a resting stage recognisable as a nucleus. Vegetative cells in old cultures, many of which must be resting, are too small for their nuclear structures to be clearly resolved and the existence in them of resting nuclei can not be excluded. Starvation, low temperatures, and various bacteriostatic drugs have so far been unable to induce the formation of resting nuclei in growing cells from young cultures. Lack of nuclei, if universal, would indeed place bacteria in an isolated position as to their taxonomy (cf. Stanier and van Niel 1941). However, it now seems that the cytology of bacterial *spores* may become a factor in the breaking down of this isolation and in the tracing of affinities to other groups of microorganisms.

2. STRUCTURE OF RESTING BACTERIAL SPORES

Ever since they were first recognised as the resting stages of bacterial cells some 70 years ago (Cohn 1876), bacterial spores have commonly been regarded as morphologically homogeneous concentrations of protoplasm, and it is only during the last fifteen years that many different observers have described discrete, Feulgen-positive, chromatinic structures in resting spores, usually in the periphery of the cell (Pietschmann and Rippel, 1932; Milovidov 1935, Stille 1937, Schaede 1939, Piekarski 1940, Stapp 1942).

In a study of the behaviour of the chromatinic structure during spore germination (Robinow 1942), I have previously described it as a single, dumbbell-shaped chromosome closely attached to the outer surface of the spore's main body of cytoplasm and somehow fitted into the narrow space between the cytoplasm and the inner surface of the outer surrounding spore membrane.

In further work, I have since confirmed my previous observation that the chromatinic body of the resting spore gives rise to the chromosomes of the vegetative cell, but its dumbbell-shape I have now recognised as the lateral aspect of a flat circular body corresponding to a nucleus rather than to a chromosome.

For my observations I have mainly used spores of two species, *B. mycoides* 1110 and *B. mesentericus*; repeated comparison with spores of *B. mycoides* Fluegge, *B. cereus*, *B. megatherium*, and *B. anthracis* has revealed essentially the same organisation. *B. mycoides* 1110 is one of the bacteriophage-producing strains of aerobic spore-forming bacteria from soil described by den Dooren de Jong (1933, 1934) who classified it as a strain of *B. mycoides* because of the selective lytic action of its phage on several typical *Mycoides* strains. The usefulness of this bacillus for cytological investigation lies in the size of its spores which are larger, on the average, than those of *B. mycoides* Fluegge and *B. cereus* although like them in every other respect.

The nuclear transformations accompanying spore formation are complex; they are the subject of a study by Dr. E. Klieneberger-

Nobel about to appear in the *Cambridge Journal of Hygiene*. In the present account I shall deal only with the finished product: the mature spore, freed from its mother cell.

In the appearance of the refractile body and the smooth contours of untreated spores there is nothing to suggest internal differentiation. The tool that helps us to gain a view of the internal organization of resting spores is dilute hydrochloric acid which we have already found of use in the study of vegetative cells where it weakens the strong affinity of the cytoplasm for basic dyes.

In the spores HCl is used for quite a different reason; it is the impermeability of the cell wall for stains which has to be overcome. Hydrochloric acid achieves this result even at room temperature while it requires heating to 60° C. to produce a significant change in the staining properties of the basophilic cytoplasm of vegetative cells.

Applied at room temperature or, for a shorter length of time, at 55–60° C., N/1 hydrochloric acid so changes the spore membrane that the interior of the cell can afterwards be stained by the same routine methods that are used for the staining of vegetative bacilli.

In the cytoplasm of hydrolysed spores, mounted in water, three concentric layers can be distinguished. The central portion, a narrow core, is refractile and stainable, and merges into a thick layer of less refractile and less readily stainable cytoplasm, bounded on its outer surface by a thin crust of stainable, non-refracting material which may be identical with the spore membrane (cf. Plate 4, figs. 22, 24). Stains tend to obscure the refractivity of the innermost layer, so that in stained preparations the more peripheral second layer appears actually more *refracting* than the more deeply stained interior. No chromatinic matter is found in the cytoplasm.

The nucleus is the most refracting element in hydrolysed spores; it is attached to the outer surface of the cytoplasm and readily distinguished from it as a brightly refractile body even in unstained water-mounted preparations. The nucleus gives a strong

Explanation of Photographs

Unless otherwise stated all photographs are from preparations that have been fixed in OsO_4 -vapour, treated with hydrochloric acid at $56-60^\circ \text{C}$ stained with Giemsa solution, and photographed mounted in water

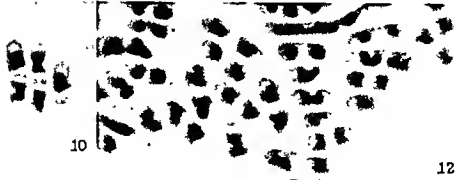
PLATE II

FIGS. 10, 11.—All figures magnified 4600 times. Some stages in the development of *B. mesentericus*. Compare the dividing chromosomes in fig. 10 with Plate I, fig. 1 g and h. Note transverse plasma membranes in fig. 10.

FIG. 12.—“Habit” picture of *Proteus vulgaris* from the edge of a colony on agar, “resting” between two periods of swarming.

FIG. 13.—“Habit” picture of *Escherichia coli* from a young, growing culture on agar.

FIGS. 14, 15.—Successive stages in the development of *B. mycoides* 1110. From a spore film incubated for $1\frac{1}{4}$ hours at 37° C. The large 4-cell bacterium in fig. 15 results from the division of the two component cells of stage (d) in the figure above. For stages earlier than (a) see Plate 4.



10

12



11

13



a-

14

15

PLATE III

FIGS. 16 a, b, c, d.—Successive division stages of the chromosomes in growing vegetative forms of *B. mycoides* Fluegge. The sharp break in the cytoplasm of the large, 4-cell bacterium in (d) is an apparent one, simulated by an unstained transverse cell wall. Cf. Plate 8, figs. 51 a, a', b. From preparations mounted in Canada balsam, magnified 4800 times.

FIG. 17 a, b.—Lateral view of peripheral nuclei in resting spores of *B. anthracis*; virulent strain "Hankow Hide."

FIG. 18.—Same as previous figure; avirulent strain.

FIG. 19 a, b.—Contracted peripheral nuclei in resting spores of *B. mesentericus*. Figs. 17-19 photographed mounted in water, magnified 3500 times. Note the smooth, unbroken contours of the cytoplasm in figs. 17-19.

FIGS. 20 a, b, and 21 a, b, c.—Electron micrographs of dried, hydrolysed resting spores of *B. mesentericus*. Magnified 7500 times; fig. 21 c magnified 15,000 times. Explanation in the text. Photographs made by Mr. G. R. Crowe of the Cavendish Laboratory, Cambridge.

16a

16b

16c

16d

17a

17b

18

20a

19a

19b

20b

21a

21c

21b

PLATE IV

Nuclei of resting spores of *B. mycoides* 1110 and their transformation during the early stages of spore formation. Unless otherwise stated the spores were hydrolysed at 60° C. and stained with Giemsa solution. All figures magnified 4600 times.

FIG. 22 a, b.—Lateral view of the nuclei in resting spores hydrolysed at room temperature for 30 minutes. (a) crystal violet, (b) Giemsa.

FIG. 23.—Peripheral nuclei in resting spores hydrolysed at 60° C. Three of the nuclei on the left are seen from the surface ("ring forms"), those in the centre from the side and those in the right bottom corner at an angle.

FIG. 24.—Lateral view of peripheral nuclei in spores incubated for 1½ minutes hydrolysed at 60° C. Structure of spores not yet noticeably different from that of resting spores. Good example of the differentiation of the cytoplasm into an inner, readily stainable and an outer less stainable layer.

FIG. 25.—Spores incubated with broth at 37° C. for 3 minutes. The nucleus has entered the cytoplasm, except in the spore in the left top corner, and has become very indistinct. Note distorted ring form in second cell from the right in the top row.

FIG. 26.—Reappearance of well defined, delicate chromatinic configurations in the cytoplasm of spores incubated for 8 minutes. Note ring forms. In the left bottom corner and towards the right retarded spores with peripheral nuclei.

FIG. 27.—Contraction of the chromatinic figures in the centre of spores which had been incubated for 12 minutes.

FIG. 28.—Further contraction and increased staining of the chromatinic structures in spores incubated for 15 minutes.

FIG. 29.—Division of the central chromatinic structure into two pairs of chromosomes in spores incubated for 55 minutes. Cf. (a) in Pl. II, fig. 14, where further development is shown.



PLATE V

FIG. 30.—Surface view of typical nuclei with peripheral concentrations of chromatin ("ring forms") in spores of *B. mycoides* 1110 incubated for 1½ minutes. The spores in Pl. 4, fig. 24 are from the same preparation. Cytoplasm out of focus. Magnified 4600 times.

FIGS. 31, 32.—Nuclei and chromosomes in germ tubes of *Penicillium notatum*. Compare with the surface views of spore nuclei in Pl. 4, fig. 23 and Pl. 5, fig. 30. Fleming's cellophane method (1944), OsO₄, HCl, Giemsa. Fig. 31 magnified 2000 times. Fig. 32 magnified 2800 times.

FIG. 33.—Chromosomes in *Cl. oedematiens* var. *gigas*, preparation by Dr. E. Klieneberger-Nobel. phot. mounted in Canada balsam. Magnified 3500 times.

FIG. 34.—Stages in the division of the central nuclear structure in *Phytomonas begoniae*. 17 days at 28° C. on dextrose potato agar. Photograph mounted in Canada balsam. Preparation by Dr. de Garcia Cabral. Magnified 3500 times.

Compare figs. 33 and 34 with the habit pictures of *Escherichia* and *Proteus*, Pl. 2, figs. 12 and 13.

FIGS. 35 a, b —Cytoplasm (a) and cell wall (b) in young vegetative forms of *B. mesentericus*. "sp," spore case. For explanation see general description of Plate 6. Magnified 3500 times.

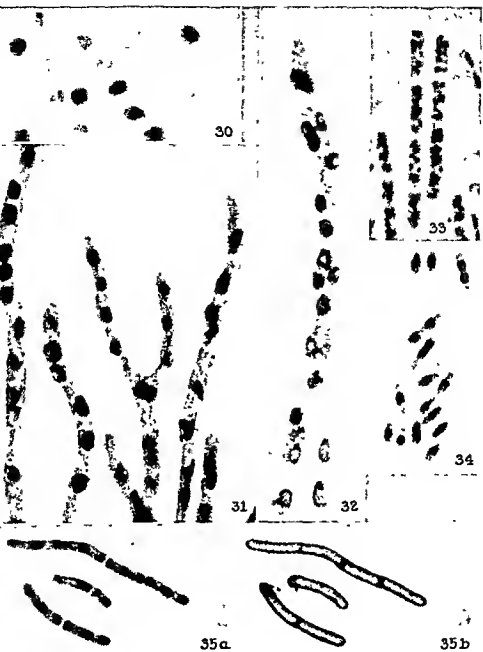


PLATE VI

In the five pairs of photographs on this plate, as well as in Plate 5, fig. 35 a, b and Plate 8, fig. 51 a, a', b, those marked "a" show only the cytoplasm, those marked "b" mainly the cell walls of one and same group of bacteria, fixed through the agar ("*in situ*") with Bouin's fluid and stained first with Giemsa solution (photographs "a") and, after extraction of this stain and mordanting with tannic acid, stained once more, this time with crystal violet (photographs "b"). The pictures are intended to illustrate the mode of formation of transverse cell walls or septa. "cb" cytoplasmic boundary; "dt" the negative images of transverse cell walls in various stages of development; "ft" a finished transverse cell wall; "ch" unstained groups of chromosomes; "sp" spore case. Figures 36-39 magnified 4600 times, figure 40 magnified 3500 times.

FIGS. 36 a, b, and 37 a, b.—*B. anthracis*, unencapsulated, avirulent strain.

FIG. 38 a, b.—*B. megatherium*. Advanced stage in the formation of the first transverse cell wall. Cf. figs. 49, 50, Pl. 8. The two sister cells still connected by a slender bridge of protoplasm.

FIG. 39 a, b.—*B. mesentericus*. Same preparation as Plate 5, fig. 35.

FIG. 40 a, b.—*B. cereus*.

dt

37a

36a

37b

40a

36b

38a

38b

ch

ft

dt

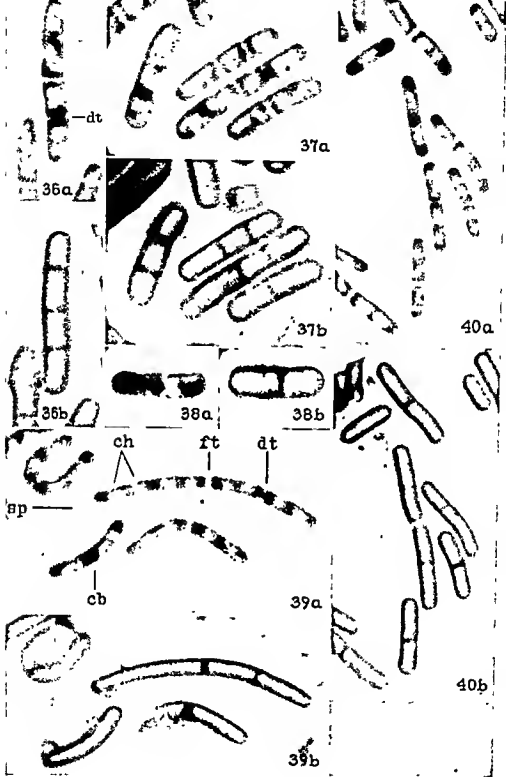
ap

cb

39a

40b

39b





positive Feulgen reaction, in sharp contrast to the negative cytoplasm, and is deeply and brilliantly stained with Giemsa solution and crystal violet (Plate 4, figs. 22, 23, 24). The simplest way of studying the nucleus is to mount a film of air-dried resting spores, which need not be fixed, in a drop of N/HCl and to examine it after an interval of some 5 to 10 minutes. After this time, the number of spores with transparent membranes, small at first, increases rapidly until cytoplasm and nucleus are visible as separate structures in most of the spores. No staining is required, but following the advice of Dr. W. J. Dowson I have obtained particularly instructive preparations by using hydrochloric acid containing 0.05 p.c. of acid fuchsin.

H. Bauer (1932) has shown, and the daily experience of cytologists everywhere has confirmed, that the shape of suitably fixed chromosomes is not significantly altered by the short treatment with N/HCl which forms part I of the Feulgen process. This is not true of the nucleus of bacterial spores, which contracts under the influence of hydrochloric acid. This contraction, which varies in extent with the species, is the cause of the highly asymmetrical shape of hydrolysed spores of *B. mesentericus*, *B. anthracis* and other species (cf. Plate 3, figs. 17, 18, 19). At 60° C. contraction is moderate in *B. mycoides* 1110, *B. mycoides* Fluegge, and *B. cereus*, but it is very evident in *B. anthracis*, *B. megatherium*, and *B. mesentericus*; in the last two species it is even conspicuous after treatment at room temperature with hydrochloric acid.

In *B. mycoides* 1110 the difference between spores treated at room temperature and at 60° C. is, on the average, the greater length of the nuclei in the former. In most spores hydrolysed at room temperature the nucleus, in optical section, is seen extending along the whole length of the spore on one side, neatly moulded to fit the cell's smooth contours and radial symmetry. In spores hydrolysed at 57-60° C. the nucleus contracts lengthwise and increases in thickness, and although it remains closely attached to the surface of the dormant cytoplasm, it now causes a hump in the formerly unbroken contours of the cell.

In anthrax spores treated at room temperature, the nucleus is

again found squeezed flat against the cytoplasm, but at 60° C. hydrochloric acid causes it to contract into a nearly spherical body protruding from the surface of the cytoplasm by as much as the diameter of the untreated spore (Plate 3, figs. 17, 18).

In *B. mesentericus*, finally, there is no difference between the effects of hydrochloric acid at room temperature and at 60° C. Protruding spherical nuclei are obtained after short treatment at either temperature (Plate 3, fig. 19).

The appearance of the nucleus varies with the degree of contraction caused by the hydrochloric acid and with the angle at which it is viewed by the observer.

In optical section, the nucleus in spores of *B. mycoides* 1110, hydrolysed at room temperature, appears bent by the curvature of the surface of the cytoplasm, it is usually thicker at one end than at the other and both ends are often thicker than the middle (Plate 4, figs. 22a, b). Through contraction (during hydrolysis at 60° C.), this lateral view is transformed into the dumbbell-shape which I have previously ascribed to the peripheral chromatic body of resting spores (Plate 4, fig. 23).

Lateral and full views alike suggest that the nucleus is a bi-concave disk with the central depression sometimes extending to part of the perimeter. The shape of the nucleus somewhat resembles that of a fresh human red blood cell. Pictures of fresh preparations of human blood in *A Textbook of Histology* by Maximow and Bloom (1938) show a strikingly wide range of shapes common to both human erythrocytes and spore nuclei. The chromatin is mainly concentrated in the periphery of the nucleus (Plate 4, fig. 23 on the left, Plate 5, fig. 30), a nucleolus has not been seen.

The same distribution is found in the compact, nearly spherical nuclei of *B. mesentericus*. In photographs from hydrolysed, Giemsa-stained preparations and in electron micrographs of hydrolysed spores of *B. mesentericus* (Plate 3, figs. 20, 21) the nuclei appear more or less homogeneous, but under the light microscope change of focus reveals that chromatinic and refractile matter are concentrated in the periphery, so that in optical

section the nuclei appear as chromatinic rings. In untreated spores they are probably as flat as those of *B. mycoides* and related species.

It is difficult to say whether the nucleus is completely isolated from the dormant cytoplasm. It might be embedded in a thin layer of cytoplasm, intervening between the outer spore membrane and the main dense body of spore cytoplasm which is destined to become the cytoplasm of the vegetative bacillus. In spores of *B. mycoides* 1110 no special "perinuclear" cytoplasm has been seen, but the fluffy contours of spore nuclei in electron micrographs of *B. mesentericus* are, perhaps, to be interpreted in this way.

The fate of the spore membrane during hydrolysis is also not clear and may vary in different species. In *B. anthracis* and *B. megatherium* the spore membrane after hydrolysis can be seen surrounding nucleus and cytoplasm, but in hydrolysed resting spores of other species usually it cannot be identified, although it is plainly visible, before and after hydrolysis, as soon as it is discarded by the young vegetative bacillus at the end of spore germination.

Some information on the spore membrane was obtained from electron micrographs of hydrolysed *Mesentericus* spores (Plate 3, figs. 20, 21). Normal resting spores possess radial symmetry. Two kinds of spores were seen in hydrolysed suspensions, both asymmetrical; in some, the asymmetry consisted merely of a slight hump in the cell's smooth and unbroken contours while in others it was due to a large round body with ragged fluffy outlines and a much lower density than that of the main body of the spore from which it protruded. The round body is the spore nucleus, and the pictures suggest that the spore membrane normally clings very tightly to the cytoplasm and that the nucleus, which lies underneath the spore membrane on the surface of the cytoplasm, contracts lengthwise under the influence of the hydrochloric acid and thereby lifts up, stretches and finally bursts through the spore membrane which, except for the hole made in it, continues firmly to adhere to the cytoplasm.

3. NUCLEAR CHANGES IN GERMINATING SPORES

The organization of the spore undergoes a dramatic change during the first few minutes of the lengthy process of germination.

To study early germination changes, ripe spores are spread out on coverslips in distilled water and dried in the incubator from 10 to 15 minutes. They are then transferred to a moist chamber, standing in the incubator at 37° C. and flooded with nutrient broth. At suitable intervals, coverslips are removed from the incubator, the broth is shaken off, the spore film fixed in osmium tetroxide vapour, rinsed in distilled water and stored in 70% alcohol. After treatment with N/HCl at 60° C. for seven to ten minutes, the spores are stained with Giemsa solution and examined mounted in water. For the staining of nuclei in germinating spores, hydrolysis has to be carried out with warm HCl because as soon as the spore prepares to germinate, it acquires the staining properties of vegetative cells.

The following description applies to spores of *B. mycoides* 1110: Shortly after the spores have been covered with warm nutrient broth, the nucleus actively enters, or is engulfed by, the cytoplasm where its outlines become very indistinct (Plate 4, fig. 25). This event is accompanied by a great increase in the (basophilic) affinity of the cytoplasm for the blue component of Giemsa's stain. The differentiation of the cytoplasm into stainable core and glassy outer layer is lost and the spore now stains blue all over. At this stage it may be difficult to trace the nucleus in spores where the cytoplasm is particularly deeply stained, but I no longer believe it disappears altogether.

In the course of the next 5 or 10 minutes there is a gradual decrease in the amount of stain absorbed by the cytoplasm, and distinct chromatinic structures reappear (Plate 4, figs. 26, 27). At first, the chromatinic structures are very delicate and look like a somewhat distorted C, S, or V; symmetrical 8-shaped configurations and elongated rings are also common. (This stage intervenes between stage D and E, fig. 52, p. 308, of my account of spore germination in *B. mycoides* Fluegge, 1942; stage C of that dia-

gram needs reinvestigation.) Gradually all these configurations contract into a thin, knotted string of chromatinic matter in the centre of the cell which further contraction (and growth?) turns into a deeply stained, compact, polygonal body (Plate 4, fig. 28). Towards the end of the first hour of incubation this body is found in various stages of division into what appears to be two pairs of chromosomes (Plate 4, fig. 29), cell division follows and further development is as outlined in the section on vegetative cells (Plate 2, figs. 14, 15). In all but the earliest stages of germination the positive result of the Feulgen test is unambiguous, the reaction being sharply confined to the chromatinic structures.

The number of chromosomes in the resting spore nucleus and the cytological significance of the transformations which the nucleus undergoes at the beginning of germination are still uncertain; and correlation of morphological and physiological observations on resting and germinating spores has not yet been attempted. The fact, however, that the biochemistry of the interaction of nucleus and cytoplasm in plant and animal cells is now being much studied, raises hopes that workers in this field will eventually provide a physiological explanation of the separation of nucleus and cytoplasm in the resting spore and of the changes in its organisation that accompany the awakening of the spore in a suitable nutrient medium.

The fact that bacteria, in their spores at least, have the particular type of nucleus described above, is of particular interest because of the light which it throws on their taxonomy. Badian (1937) pointed out that yeasts resemble bacteria in the size and shape of their chromosomes, and this has been my experience also; but the chromosomes of yeast cells are notoriously difficult to stain and I think that until staining techniques have improved, the similarity between the *nuclei* of bacterial spores and those of yeasts and certain moulds is more convincing.

In yeasts, nuclei with ring- or crescent-shaped peripheral concentrations of chromatin have been described several times (Rochlin 1933, Badian 1937, P. T. Thomas unpublished 1943), and, using the HCl-Giemsa method, I have also found them in *Peni-*

cillium, *Eurotium herbariorum*, and *Pullularia pullulans* de Bary (cf. Plate 5, figs. 30, 31, 32). At present the similarity extends only to the arrangement of the chromatin; the question of a nucleolus is still unsettled in bacteria. Further discussion of these still very fragmentary observations seems unjustified.

Enough, perhaps, has been said to show that the study of the nuclear structures of bacteria promises to be a fruitful one for cytologists as well as biochemists and taxonomists.

4. CELL DIVISION IN THE GENUS BACILLUS

It has been known for fifty years or more, though it is often forgotten, that many bacteria from young cultures—the aerobic spore-formers, for instance, and perhaps even certain cocci—are already divided into two compartments by a median transverse septum, continuous with the outer supporting cell wall, long before there are any signs of fission (“division”) of the bacterium. The HCl-Giemsa method and other procedures, to be described presently, have further shown that the protoplasts in the two compartments on either side of the median transverse cell wall just mentioned, are in turn in a more or less advanced stage of division and that it would be correct to say that the majority of rod-shaped bacteria from young cultures, far from being single cells, are really “two-cells about-to-become-four.”

One of the earliest detailed descriptions of cell division in a filamentous bacterium was given by Migula (1894, 1897) for *B. oxalaticus*. This organism, of unknown habitat and source, apparently had the distinction of possessing a central sap vacuole. The salient points in Migula's account which begins shortly after spore germination are these: As the growing cell elongates, the sap vacuole is gradually cut in two by the centripetal growth of a ring-shaped bulge arising in the peripheral cytoplasm. During the continued elongation of the bacillus, the formation of plasma rings is repeated several times but there is *no formation of transverse cell walls*. Much later (*erst viel später*) annular ridges grow out from the inner surface of the cell wall at the base of the first

formed (oldest) plasma rings and, growing centripetally, form first annular diaphragms and finally delicate transverse cell walls. Migula concludes his description: "*Usually the cells remain contiguous for a considerable time thus forming filaments of varying length, with several transverse cell walls.*" No mention is made of an eventual constriction of the cell wall which would lead to the division of the bacterium as a whole. In the first of the two series of drawings of cell division in *B. oxalaticus* published by Migula in 1894 and 1897, a slight constriction of the cell wall is actually seen near the middle of the longest composite filament (fig. 12); none appears in the much longer filament which is last in the second series (fig. 1, 1). (Italics are mine; since my translation of the relevant passages in Migula's paper differs from that given by Knaysi 1941, readers are advised to consult the original German version.)

Where no distinction is drawn between cell wall and plasma membrane, division of rod-shaped bacteria ("cells") is usually described as being by simple constriction.

Knaysi (1941), working chiefly with *B. cereus*, concluded that cell division in rod-shaped bacteria follows neither the course outlined by Migula for *B. oxalaticus*, nor can it be adequately described as a simple constriction but that bacteria divide by a retraction of the cytoplasm in opposite directions from the middle plane, followed by the formation of two cell walls which are separate from the beginning.

My own observations which support Migula's rather than Knaysi's findings were made on *B. mesentericus*, *B. anthracis*, *B. cereus*, *B. mycoides*, and *B. megatherium*, and also on *Escherichia coli*.

Cell wall and cytoplasm of bacteria differ so widely in their staining properties that it is difficult optimally to stain both by the same process. In trying to overcome this obstacle I have found it helpful to stain separately, in two steps, first the cytoplasm and then the cell wall of the same group of bacteria with appropriate methods and to superimpose the two pictures. To avoid shrinkage and distortion, growing bacteria are fixed *in situ* through the agar

with Bouin's fluid (E. Klieneberger 1934, 1942; Rohinow 1944). The cytoplasm is next stained lightly, but very distinctly, by floating the coverslip bearing the film of fixed bacteria for 10 to 15 seconds on Giemsa stain; (2 drops of Gurr's R. 66 in every ml. of water). The film is then mounted in a drop of water, sealed with wax and a suitable field photographed (Plate 6, photographs "a"). The preparation is then removed from the slide, decolourized with acid alcohol and after the method of Gutstein (1924) mordanted for 20-30 minutes with 5-10 p.c. tannic acid in water. This reagent greatly reduces the affinity of the cytoplasm for crystal violet, the stain which is next used to colour the cell wall; 5-10 seconds on 0.02 p.c. crystal violet in water proved ample for this. The same group of cells is then identified and photographed once more. No attempt was made to stain the slime layer or the capsule.

Two very different aspects of the bacteria are obtained with the two methods. After the first staining (photographs "a," Plate 6) the cytoplasm appears banded by alternating dark and light areas; in some places it is constricted by annular furrows of varying depth, in others its continuity is interrupted by sharply drawn rectilinear transverse gaps. The nuclear structures are not stained during the brief sojourn of Giemsa solution and their negative images appear as white irregular patches in the light blue cytoplasm. Thus, the bacteria have assumed a most unnatural appearance unlike any picture seen when the same organisms are examined alive by direct or dark ground illumination.

After the treatment with tannic acid and crystal violet, which follows the first staining procedure, a much more normal aspect is presented by the same group of bacteria (photographs "b," Plate 6). The cell wall is now distinctly stained and rod-forms and filaments have reacquired the wholeness and the smooth contours which they had in life. The notches, gaps and constrictions, conspicuous in the Giemsa preparation, have now disappeared and are found to have been simulated by unstained, and hence invisible, transverse cell walls in various stages of development.

Bouin-Giemsa preparations (photographs "a" on Plate 6) show that transverse cell walls develop in certain regions of the cyto-

plasm which stain very deeply. These dark regions coincide with the sites of much narrower, delicate transverse lines in the cytoplasm which in OsO_4 -HCl-Giemsa preparations intervene at regular intervals between groups of nuclear structures and which represent transverse plasma membranes or *cell boundaries* (cf. Plate 2, figs. 10, 11, 14 and 15; Plate 3, fig. 16d).

In a given rod or filament, the number of cytoplasmic cell boundaries is usually greater than the number of transverse cell walls, completed, or in process of formation. In other words, the formation of cytoplasmic cell boundaries precedes that of transverse cell walls; several boundaries may be laid down in a bacillus before the first of them is replaced by a transverse cell wall.

A newly formed transverse cell wall may split immediately and thus achieve the division (by constriction) of the whole bacterium or it may remain unchanged for a relatively long time; in the latter event several additional transverse cell walls may be formed before the bacillus, which has meanwhile grown into a long septate filament, shows any signs of division or fragmentation.

Differences in mode of growth and multiplication, e.g., between *B. anthracis* (Plate 6, figs. 36, 37) and *B. mesentericus* (Plate 5, fig. 35, and Plate 6, fig. 39) may thus be reduced to differences in the time relationship of the following distinct processes:

- (1) *Division of the cytoplasm*; completed by the formation of a transverse plasma membrane or cell boundary and eventually followed by
- (2) the formation of a *transverse septum*, continuous with the outer supporting cell wall.
- (3) *The splitting (delamination) of transverse cell walls* leading to the division (by constriction) of whole bacteria and the fragmentation of filamentous forms.

Let us briefly reconsider these points separately:

1. *Transverse Plasma Membranes or Cytoplasmic Boundaries* have previously been described in *B. mycoides* by Guilliermond (1908), in *B. subtilis* by Knaysi (1930) and in *B. mycoides*, *B. mesentericus*, and *Escherichia coli* by Robinow (1942, 1944). Cell boundaries are very distinct in OsO_4 -HCl-Giemsa preparations

of aerobic spore formers, but invisible or indistinct in preparations of *Escherichia* and *Protocus* stained by this method. The best way to demonstrate them in *Escherichia* is by brief staining with methylene blue or Giemsa solution, following Bouin fixation. In this way it has been found that in bacteria from young cultures of *Escherichia coli* and *Protocus vulgaris*, previously described as multinucleate single cells (Piekarski 1937, Neumann 1941), boundaries intervene between recently divided chromosomes in the same way as in the aerobic spore-formers.

A rod-form of *Escherichia* consists of two cells, products of an internal division which is completed before constriction of the bacterium begins, and usually the two halves of a dividing bacterium are themselves already in a more or less advanced stage of cell division (text fig. 1). Formation of the first transverse cell wall apparently coincides with the fission of the whole bacterium. In this respect *Escherichia*, *Protocus*, and *Salmonella* differ from the aerobic spore-formers where the first transverse cell wall is laid down well in advance of the division of the whole bacteria.

The single cells that characterise old cultures of *Escherichia* are the result of a slowing down of the rate of cell division relative to the rate of fission ("division") of whole bacteria.

2. *Transverse septa continuous with the outer supporting cell wall (Transverse cell walls).*

Well known to the older bacteriologists, e.g., de Bary 1884, L. Klein 1889, Migula 1894, 1897, in whose exquisite drawings they frequently appear, they have more recently been described by Knaysi (1930) and Bisset (1939).

Transverse cell walls are very conspicuous in *B. megatherium*. In staining them, a second, seemingly barbarous method proved helpful in addition to the tannic acid-crystal violet technique. The experience of Ruhland and Hoffmann (1925) with *Beggiatoa mirabilis* suggested that alkali might loosen the normally very close contact of cytoplasm and cell wall. After several unsuccessful attempts, a boiling solution of 2.5-8.0% sodium hydroxide in water applied to air-dried but otherwise unfixed impression preparations proved satisfactory. This treatment causes a more or less

extensive retraction of the cytoplasm from the cell wall and thus gives a clear view of the transverse septa. In some of the "plasmolysed" bacteria the cell wall is thrown into folds, but in most the outlines and dimensions have remained fairly normal (cf. Plate 7, figs. 41, 42).

It is important to realise that the transverse septa arise from the inner surface of the cell wall and that their development is not accompanied by changes in the outlines of the bacterium. The two-step separate staining of cytoplasm and cell wall illustrated by Plates 5, 6, and 8, figs 35, 40, and 51, shows clearly that continuity of the cytoplasm is preserved to the last during the gradual inward advance of a developing transverse cell wall. I am thus forced to disagree with the picture of cell division in bacteria recently outlined by Knaysi (1941) whom I believe to have been deceived by various kinds of artefacts. Mudd, Heinmets and Anderson (1943) have shown in electron micrographs that in the *Pneumococcus* the cytoplasm is normally closely applied to the cell wall and that the latter, which the authors regard as solid, is in turn surrounded by a gel of low density, the capsule. My own observations on the genus *Bacillus* show that here, too, the cytoplasm is everywhere in close contact with the cell wall. Observations made with dark ground illumination, silver preparations, and electron micrographs suggest in addition that, like the pneumococcus, some bacilli are surrounded by a sharply defined capsule, thicker, but much more delicate than the cell wall. The capsule is only on the outside of the bacterium and does not follow the transverse septa into the interior of the organism. Further work is required before it will be possible satisfactorily to correlate the results of ordinary staining methods and the ideas on the relationship of cytoplasm and cell wall that have been derived from the many electron micrographs of bacilli published in recent years.

It seems probable that the plasma bridges (rings) which Migula described as dividing the central vacuoles in growing filaments of *B. oxalaticus* at regular intervals, correspond to the cytoplasmic cell boundaries of the present investigation. His description of the

development of transverse cell walls, too, is in agreement with my own observations. It is tempting to regard the central vacuole in *B. oxalaticus* as an unstained nuclear structure, but I think Migula's own experiments have definitely disproved this idea; the chromosomes were presumably contained in the dense peripheral cytoplasm.

It remains to mention the controversial question of the nature of the process which leads to the splitting of transverse cell walls and thus to the division, not always equal, of a bacterium or cell chain. Sooner or later a transverse septum must needs acquire double structure but whether it is double from the beginning or first laid down in single thickness I have nowhere been able to decide with certainty.

A large scale model of the formation of transverse cell walls in bacteria is provided by *Schizosaccharomyces Pombe* (Plate 7, figs. 44-47). (For a different opinion see Knaysi 1941.) The centripetal advance of a transverse septum, beginning with a sharp ridge on the inner surface of the cell wall, is more easily studied in this organism than in the largest bacteria. Bright field observations confirmed the reality of the appearance seen in the fixed and stained preparations. Cytoplasmic cell boundaries or cell plates which, in bacteria, are usually laid down before the formation of transverse cell walls were not observed in the fission yeast. The dark line across the corner cell in Plate 7, fig. 44, is not the optical section of a preformed cytoplasmic boundary but a groove in the peripheral cytoplasm caused by the advancing cell wall. The folds in the wall of some of the cells in Plate 7, figs. 44-47, are due to fixation; they enhance the irregular, wavy outlines which are natural to *Schizosaccharomyces Pombe* (cf. A. Joergensen (1911) for drawings of the closely related species *Sch. mellacei*).

The view for which we have adduced evidence, that rod-forms from young cultures of bacteria are not homologous with single cells, is not new. It was held by Migula, 1897 (*vide* pp. 142 and 171, Vol. 1 of his *System der Bakteriologie*) and his contemporaries, and is implicit in many observations published since; e.g.,

in A. Fischer's well known description of the development of *B. subtilis* in his *Vorlesungen über Bakteriologie*, 1903).

The coccoid nature of the true elementary cell of *B. megatherium* is very evident during the earliest stages of spore germination (Plate 8, fig. 48). When the young bacterium becomes rod-shaped (Plate 8, figs. 49 and 50) it has already experienced an externally invisible cell division.

In *B. megatherium* and related forms, there must be something in the structure of the outer supporting cell wall which ensures that growth and division of the individual coccoid elements shall proceed always in one and the same single dimension of space. Whatever its nature, this structural principle is not unsbakeable, as is shown by the curious behaviour of *B. megatherium* developing on 2% malt agar where a weakening of the usual rigid polarity leads to the formation of sarcina-like clusters and unusual two-dimensional cell configurations (Plate 8, figs. 52, 53).

Clearly, the rod-shape of bacteria should not be accepted as an irreducible morphological fact but should be regarded as a complex phenomenon, to be analysed experimentally.

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